Ultraschallvokalisationen bei Maus und Ratte

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Kommunikative Signale des motivational-affektiven Zustands?

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INHALT

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- ÜBERSICHT

Studie I:

Wöhr, M. & Schwarting, R.K.W. (2008). Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behavioral Neuroscience*, 122 (2), 310-330.

Studie II:

Wöhr, M., Dahlhoff, M., Wolf, E., Holsboer, F., Schwarting, R.K.W. & Wotjak, C.T. (accepted manuscript). Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: An embryo-transfer study. *Behavior Genetics*.

Studie III:

Wöhr, M., Houx, B., Schwarting, R.K.W. & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology & Behavior*, 93 (4-5), 766-776.

Studie IV:

Wöhr, M. & Schwarting, R.K.W. (2007). Ultrasonic communication in rats: Can playback of 50-kHz calls induce approach behavior? *PLoS ONE*, 2 (12), e1365.

Studie V:

Sadananda, M., **Wöhr, M.** & Schwarting, R.K.W. (2008). Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. *Neuroscience Letters*, 435 (1), 17-23.

Studie VI:

Wöhr, M. & Schwarting, R.K.W. (in press). Ultrasonic calling during fear conditioning in the rat: No evidence for an audience effect. *Animal Behaviour*.

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* Anmerkung: Teile dieser Studie sind in der Diplomarbeit von Markus Wöhr enthalten. Es handelt sich hierbei um den Zusammenhang zwischen maternaler Fürsorge und Emission isolations-induzierter Ultraschallvokalisationen [*ultrasonic vocalizations*] sowie um die Prüfung, inwiefern isolations-induzierte Ultraschallvokalisationen maternales Suchverhalten stimulieren können. Neu, das heißt nicht in der Diplomarbeit enthalten, ist der Zusammenhang zwischen maternaler Fürsorge und adulten Verhaltensweisen, wie lokomotorische Aktivität in der Aktivitätsbox [*activity box*], angst-ähnlichem Verhalten im erhöhten Pluslabyrinth [*elevated plus maze*], und furcht-ähnlichem Verhalten während der Furchtkonditionierung [*fear conditioning*]. Ferner hinzugekommen ist die Prüfung der prädiktiven Validität von isolations-induzierten Ultraschallvokalisationen hinsichtlich der Vorhersage dieser eben genannten adulten Verhaltensweisen.

EINLEITUNG

Seit über einem halben Jahrhundert ist bekannt, dass Mäuse (Zippelius & Schleidt, 1956) und Ratten (Anderson, 1954) über die Fähigkeit verfügen, Ultraschallvokalisationen [*ultrasonic vocalizations*] auszusenden. Für die biopsychologische Grundlagenforschung stellt das Rufverhalten dieser Tiere ein hilfreiches Werkzeug dar, da diese Rufe in motivational relevanten Situationen, wie Trennung eines Jungtiers von der Mutter [*isolation*], Paarung [*mating*] und Kampf [*fighting*] auftreten (zur Übersicht siehe: Constantini & D'Amato; Hofer, 1996; Knutson et al., 2002; Portfors, 2007).

In Abhängigkeit von Alter und motivationalem Zustand des Tieres können bei der Ratte drei verschiedene Typen der Ultraschallvokalisation unterschieden werden. Jungtiere senden in für sie potentiell lebensbedrohlichen Situationen, wie zum Beispiel in Folge der Trennung von Mutter und Geschwister, 40-kHz Vokalisationen aus (Allin & Banks, 1971; Hofer & Shair, 1978), was bei der Mutter Such- und Eintrageverhalten [*retrieval behavior*] induziert (Allin & Banks, 1972). Juvenile und adulte Tiere emittieren 22-kHz und 50-kHz Rufe. Die 22-kHz Rufe treten in aversiven Situationen auf, wie zum Beispiel bei Konfrontation mit einem Fressfeind (Blanchard et al., 1990; 1991; 1992; Shepherd et al., 1992; zur Übersicht siehe: Litvin et al., 2007) oder im innerartlichen Kampf (Kaltwasser,

1990a; Kroes et al. et al., 2007; Lehman & Adams, 1977; Lore et al, 1976; Sales 1972a; Thomas et al., 1983). Dahingegen sind 50-kHz Rufe charakteristisch für appetitive Situationen. Sie können etwa während dem Spiel [*rough-and-tumble play*] junger Ratten (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998) oder beim Paarungsverhalten geschlechtsreifer Tiere beobachtet werden (Barfield et al., 1979; Bialy et al., 2000; Burgdorf et al., im Druck; Geyer & Barfield, 1978; McGinnis & Vakulenko, 2003; McIntosh et al., 1978; Sales, 1972b; White & Barfield, 1990; White et al., 1990). Auch das Kitzeln [*tickling*] des Tieres durch den Versuchsleiter hat sich als effektive Methode erwiesen, um 50-kHz Vokalisationen auszulösen (Burgdorf & Panksepp, 2001; Burgdorf et al., im Druck; Mällo et al., 2007; Panksepp & Burgdorf, 1999; 2000; 2003; Schwarting et al., 2007).

Die Maus verfügt über ein ähnliches Repertoire an Ultraschallvokalisationen. Wie auch junge Ratten senden junge Mäuse in Reaktion auf eine Trennung von Mutter und Geschwister Ultraschallvokalisationen aus (Zippelius & Schleidt, 1956), wodurch sie maternales Such- und Eintrageverhalten induzieren (Sewell, 1970). Wahrscheinlich durch die geringere Körpergröße bedingt, weisen diese Vokalisationen jedoch eine höhere Frequenz auf als jene, welche die junge Ratte emittiert (Hashimoto et al., 2004). So liegen die Vokalisationen der Maus etwa bei 60 kHz (Hashimoto et al., 2004). Ein gewichtiger Unterschied zwischen Maus und Ratte ist jedoch, dass adulte Mäuse keine den 22-kHz Vokalisationen entsprechenden Rufe produzieren (Blanchard et al., 2001). Es konnten aber in Situationen, in denen bei der Ratte 50-kHz Vokalisationen zu beobachten sind, auch bei Mäusen Ultraschallvokalisationen nachgewiesen werden, wie zum Beispiel bei der Paarung (Nyby, 1983; Sales, 1972b; White et al., 1998). Diese Vokalisationen weisen eine Frequenz von etwa 70 kHz auf (Holy & Guo, 2005).

Bislang ist verhältnismäßig wenig über die biologische Funktion dieser Vokalisationen bekannt, obwohl schon früh postuliert wurde, die Ultraschallvokalisationen würden der innerartlichen Kommunikation [*communication*] dienen (Sales & Pye, 1974; Smith, 1979). Unter Kommunikation wird ein Austausch von Informationen verstanden, bei der durch ein Signal eine Veränderung im Empfänger ausgelöst wird, wobei entweder die Bereitstellung der Information dem Sender oder der Zugang zur Information dem Empfänger einen Nutzen bringen muss (Bradbury & Vehrencamp, 1998).

Häufig wird eine kommunikative Funktion akustischer Reize durch das künstliche Abspielen [*playback*] dieser überprüft (Allin & Banks, 1972; Bang et al., im Druck; Brudzynski & Chiu, 1995; Brouette-Lahlou et al., 1982; Burman et al., 2007; Ehret, 1992; Ehret & Haack, 1981; 1982; Endres et al., 2007; Geyer et al., 1978; McIntosh et al., 1978;

Lindquist et al., 2004; Pomerantz et al., 1983; Sales, 1991; Sewell, 1970; Smotherman et al., 1974; Tankhiwale et al., 2007; Terkel et al., 1979; White & Barfield, 1990; White et al., 1993; zur Übersicht siehe: Owings & Morton, 1998). Hierbei werden zuvor elektronisch aufgezeichnete akustische Reize unter streng kontrollierten Bedingungen abgespielt und die Reaktion des Empfängers erfasst. Entscheidend ist hierbei, dass der Sender nicht anwesend ist, so dass als Ursache für eine Verhaltensänderung des Empfängers zwischen dem akustischen Signal und den die Signalemission normalerweise begleitenden Verhaltensweisen des Senders dissoziiert werden kann.

Diesen Abspielexperimenten gehen in der Regel umfassende Verhaltensbeobachtungen voraus, welche eine genaue Beschreibung des Auftretenszeitpunkts potentieller akustischer Signale, sowie der hierdurch möglicherweise im Empfänger ausgelösten Verhaltensänderungen, zum Ziel haben (Blanchard et al., 1991; Brewster & Leon, 1980; Lehman & Adams, 1977; Lore et al., 1976; Moles & D'Amato, 2000; Moles et al., 2007; Panksepp et al., 2007; Sales, 1972a; Zippelius & Schleidt, 1956; zur Übersicht siehe: Owings & Morton, 1998).

Ein anderer Ansatz, eine potentielle Signalfunktion akustischer Reize experimentell zu überprüfen, wird in Studien verfolgt, in welchen der Sender, beispielsweise durch Durchtrennung der die Stimmbänder innervierenden Nerven, an der Emission von Vokalisationen gehindert wird (Hashimoto et al., 2001; Hofer & Shair, 1993; Thomas et al., 1983; Pomerantz, 1983; Wang et al., 2008). Umgekehrt wird in anderen Untersuchungen der Empfänger, beispielsweise durch Verschluss des Gehörgangs, am Empfang der Vokalisationen gehindert (Siviy & Panksepp, 1987; Thomas et al., 1983).

Ultraschallvokalisationen bei der Ratte
- 40-kHz Vokalisationen

40-kHz Vokalisationen und affektiver Zustand

In potentiell lebensbedrohlichen Situationen emittieren junge Ratten 40-kHz Vokalisationen. So können diese Rufe in Reaktion auf die Trennung von Mutter und Geschwister (Allin & Banks, 1971; Hofer & Shair, 1978) sowie bei einem Abfall der Körpertemperatur beobachtet werden (Blumberg & Skoloff, 2001). Ausgehend von der ersten Beobachtung wird angenommen, dass diese Vokalisationen einen negativen affektiven Zustand reflektieren, wie etwa Angst (Hofer, 1996).

Basierend auf der zweiten Beobachtung wurde postuliert, dass die Vokalisationen ein Nebenprodukt eines die Thermoregulation unterstützenden physiologischen Prozesses darstellen (Blumberg & Skoloff, 2001). Demnach handelt es sich bei der Ultraschallemission junger Nagetiere um ein akustisches Nebenprodukt eines durch erniedrigte Temperatur induzierten komplexen physiologischen Mechanismus, welcher mit einer veränderten Atmung einhergeht (Blumberg & Sokoloff, 2001). Dieser, als abdominale Kompressionsreaktion bezeichnete, physiologische Mechanismus diene dazu den venösen Blutumsatz der veränderten Temperatur anzupassen. Tatsächlich konnte nachgewiesen werden, dass Umgebungstemperatur und die Emission von 40-kHz Rufen negativ korreliert sind (Allin &

Banks, 1971). Außerdem konnten Hofer und Shair (1992) zeigen, dass junge Ratten, die aus einer kälteinduzierten Bewusstlosigkeit erwachen, mit ihren ersten Atemzügen Ultraschall aussenden und weniger Zeit benötigen ihre normale Körpertemperatur wieder zu erreichen als Ratten, welchen operativ, mittels Durchtrennung der die Stimmbänder innervierenden Nerven, die Möglichkeit genommen wurde zu vokalisieren. Darüber hinaus sterben devokalisierte Tiere eher an Unterkühlung als Tiere, welche nicht devokalisiert wurden (Hofer & Shair, 1993).

Gegen die Annahme, dass 40-kHz Vokalisationen lediglich ein physiologisches Artefakt eines Thermoregulationsprozesses darstellen, sprechen jedoch Untersuchungen, die zeigen konnten, dass Jungtiere bereits ab dem dritten Lebenstag weniger 40-kHz Rufe in Anwesenheit eins weiteren Jungtiers aussenden - auch wenn dieses Jungtier narkotisiert und auf die Umgebungstemperatur abgekühlt ist und daher keine Wärme spenden konnte (Carden & Hofer, 1992). Ab der zweiten Lebenswoche erscheint dann die soziale Isolation und nicht länger die Absenkung der Körpertemperatur von primärer Bedeutung zu sein. Ab diesem Zeitpunkt nämlich vokalisiert das Jungtier auch bei einer unter Nesttemperatur durchgeführten Isolation (Carden & Hofer, 1992). Auch ein Zusammenhang von Temperatur und Ultraschallvokalisation konnte ab diesem Zeitpunkt nicht mehr beobachtet werden (Brunelli et al., 1996; Brunelli, 1997). Soziale Faktoren scheinen also im Zuge der Entwicklung des Tieres an Bedeutung für die Produktion von 40-kHz Rufen zu gewinnen, wohingegen die Bedeutung der Temperatur abzunehmen scheint.

Im Sinne der affektiven Hypothese konnte ferner gezeigt werden, dass die Auftretenshäufigkeit von 40-kHz Vokalisationen pharmakologisch moduliert werden kann. So konnten Gardner (1985) und Insel et al. (1986) zeigen, dass anxiolytische Substanzen, wie das Benzodiazepin Diazepam, das Rufverhalten inhibieren ohne das motorische Verhalten zu beeinflussen, wohingegen anxiogene Substanzen, wie Pentylenetetrazol, das Rufverhalten steigern. Ferner hemmen auch Serotoninwiederaufnahmehemmer, wie Clomipramin, Paroxetin und Citalopram, das isolations-induzierte Rufverhalten (Winslow & Insel, 1990). Potente Inhibitoren des isolations-induzierten Rufverhaltens sind darüber hinaus Opioid-Agonisten, wie Morphium (Carden et al., 1996). Zusammenfassend folgerte Hofer (1996) daher, dass es jene Substanzen sind, die sich bei der Behandlung von Angststörungen beim Menschen als hilfreich erweisen haben, die das Rufverhalten isolierter Ratten inhibieren.

Die affektive Hypothese wird außerdem durch Zuchtstudien gestützt. Tiere, die selektiv auf gering oder stark ausgeprägtes Rufverhalten gezüchtet wurden, unterschieden sich in ihrem angst-ähnlichem Verhalten im Erwachsenalter (Brunelli & Hofer, 2007; Brunelli,

2005; Brunelli et al., 1997; 2001; 2002; Hofer et al., 2001; Shair, 2000; Zimmerberg et al., 2005). Adulte Tiere, welche der stark vokalisierenden Linie entstammen, zeigten mehr angst-ähnliches Verhalten im Offenfeld [*open field*] und mehr depressions-ähnliches Verhalten beim erzwungenen Schwimmen [*forced swim test*] als Tiere der niedrig vokalisierenden Linie (Brunelli, 2005; Shair et al., 2000; Zimmerberg et al., 2005). Auch emittierten die ersteren in Reaktion auf eine Handhabung [*handling*] mehr 22-kHz Vokalisationen als die letzteren (Brunelli, 2005; Shair et al., 2000). Begleitet werden diese Unterschiede im Verhalten von zahlreichen physiologischen Veränderungen, wie etwa bezüglich der Herzrate in aversiven Situationen oder Neurotransmitterkonzentrationen in bestimmten Hirnregionen (Brunelli, 2005; Brunelli & Hofer, 2007; Hofer et al., 2001; Zimmerberg et al., 2005). Kritisch muss allerdings angemerkt werden, dass sich die beiden Linien nicht, wie im Rahmen der affektiven Hypothese zu erwarten, hinsichtlich des Sozialverhaltens oder der in Reaktion auf einen elektrischen Schock gezeigten Verhaltensstarre [*freezing*] unterscheiden (Zimmerberg et al., 2005). Schließlich sind die Ergebnisse hinsichtlich des adulten angst-ähnlichen Verhaltens auf dem erhöhten Pluslabyrinth [*elevated plus maze*] widersprüchlich (Dichter et al., 1996; Rojowski et al., 1999; Shair et al., 2000) – was insofern schwerwiegend ist, als dass es sich beim erhöhten Pluslabyrinth um den wohl verbreitesten Test zur Messung unkonditionierter Angst handelt (Carobrez & Bertoglio, 2005). In nicht selektiv gezüchteten Tieren konnte gar eine negative Korrelation zwischen 40-kHz Vokalisationen des Jungtiers und dem im Erwachsenenalter gezeigten angst-ähnlichen Verhalten im Pluslabyrinth beobachtet werden (Schwarting & Pawlak, 2004). Jüngst wurde daher die affektive Hypothese erweitert und die Bedeutung verschiedener Strategien der Stressbewältigung [*coping*] in Reaktion auf aversive Situationen hervorgehoben (Brunelli & Hofer, 2007). So können Organismen beispielsweise mit aktiven oder mit passiven Verhaltensweisen auf eine Bedrohungssituation reagieren.

In Übereinstimmung mit der affektiven Hypothese unterscheiden sich jedoch auch Tiere, die nach hohem beziehungsweise niedrigem angst-ähnlichen Verhalten im Erwachsenenalter gezüchtet wurden, in ihrer isolations-induzierten Ultraschallvokalisation. Dies trifft beispielsweise auf Ratten zu, welche gemäß stark oder gering ausgeprägtem angst-ähnlichen Verhalten auf dem erhöhten Pluslabyrinth gezüchtet wurden. So emittierten Ratten, die der hoch-ängstlichen Zuchtlinie entstammten, mehr 40-kHz Vokalisationen in Reaktion auf eine Trennung als Ratten der niedrig-ängstlichen Zuchtlinie (Wigger et al., 2001). Ähnliche Beobachtungen konnten an Tieren gewonnen werden, welche gemäß Lokomotion und Defäkation gezüchtet wurden (Insel & Hill, 1987; Naito et al., 2000).

Zusammenfassend kann daher geurteilt werden, dass die affektive Hypothese nicht allein durch pharmakologische Studien, sondern auch durch prospektive Längsschnittstudien unter Verwendung selektiv gezüchteter Tiere gestützt wird. Eine Berücksichtigung unterschiedlicher Stressbewältigungsstrategien erscheint angebracht.

Signalfunktion von 40-kHz Vokalisationen

Bereits Zippelius und Schleidt (1956) konnten in jener Studie, in welcher sie isolations-induzierte Ultraschallvokalisationen bei Nagern erstmals beschrieben, Belege dafür erbringen, dass es sich bei diesen Ultraschallrufen um ein kommunikatives Signal handelt. Sie beobachteten, dass lediglich vokalisierende Mäuse von der Mutter in das Nest eingetragen wurden, nicht jedoch jene, die narkotisiert wurden und daher nicht vokalisieren konnten. Den experimentellen Nachweis, dass es sich bei dem kritischen Stimulus auch bei Ratten um die Ultraschallvokalisationen handelt, lieferten Allin und Banks (1972). Sie konnten nachweisen, dass Rattenmütter auf die Darbietung von auf Tonband aufgenommener Ultraschallrufe mit Verlassen des Nestes und Suchverhalten reagierten. Allerdings verließ lediglich die Hälfte der Mütter mindestens einmal von sechs Rufdarbietungen das Nest. Es wurde daher von Smotherman et al. (1974) postuliert, das Eintrageverhalten werde von akustischen und olfaktorischen Stimuli geleitet. Akustische Stimuli alleine seien nicht ausreichend zur Induktion von Eintrageverhalten in der Ratte. Tatsächlich konnten Smotherman et al. (1974) keine Präferenz für jenen Arm eines Y-Labyrinths beobachten, in welchem aufgezeichnete Ultraschallvokalisationen dargeboten wurden, gegenüber einem Arm ohne Tondarbietung. Wurden jedoch sowohl akustische als auch olfaktorische Stimuli dargeboten, so präferierten die Mütter diesen Arm gegenüber einem Arm in dem ausschließlich entweder akustische oder olfaktorische Stimuli dargeboten wurden. Es wurde daher angenommen, dass olfaktorische Stimuli für die Stimulation des Suchverhaltens entscheidend sind, und dass akustische Stimuli die Lokalisation des Jungtieres erleichtern. Tatsächlich gibt es überzeugende Belege für die Bedeutung akustischer Stimuli für die Lokalisation des Jungtieres. So zeigen Untersuchungen mit auf Tonband aufgenommer Rufe, dass Mütter die Geräuschquelle sehr genau orten können. In einer Untersuchung von Allin & Banks (1972) suchten die Mütter in 80 % der Fälle in jenem von sechs Feldern in dem das Geräusch dargeboten wurde. Ähnliche Befunde liegen für Vokalisationen vor, welche in Folge einer kälteinduzierten Bewusstlosigkeit auftreten (Brunelli et al., 1994).

Doch auch hinsichtlich der im Nest gezeigten maternale Pflege [*maternal care*] scheinen die 40-kHz Vokalisationen wichtig zu sein. So konnte beobachtet werden, dass die

Emission dieser Rufe Nestbauaktivitäten der Mutter erhöht (Hashimoto et al., 2001; Noirot, 1972) und die Latenz reduziert bis ein bedrohtes Nest von der Mutter in Sicherheit gebracht wird (Brewster & Leon, 1980). Außerdem scheinen sie maternales Lecken im Anogenitalbereich [*anogenital licking*] der Jungtiere zu fördern (Brouette-Lahlou et al., 1992) und die Ausschüttung von Prolaktin zu stimulieren (Hashimoto et al., 2001; Terkel et al., 1979). Die Prolaktinausschüttung korreliert positiv mit maternalen Verhaltensweisen, wie Nestbau und der Zeit, die benötigt wird, um Jungtiere in das Nest einzutragen (Hashimoto et al., 2001).

Darüber hinaus scheint jedoch umgekehrt auch die maternale Pflege einen Einfluss auf das Rufverhalten der Jungtiere zu haben. Dies verdeutlicht die Tatsache, dass Kontakt mit der Mutter mit einer raschen Reduktion der Rufemission einhergeht (Hofer, 1996; Hofer & Shair, 1978). Weiteres prominentes Beispiel für die Modulation der Auftretenshäufigkeit von Ultraschallvokalisationen im Jungtier durch die Mutter ist das Phänomen der maternalen Potentierung [*maternal potentiation*]. Wird einem Jungtier in Folge einer Isolation ein kurzer Kontakt mit der Mutter gewährt, so liegt die Auftretenshäufigkeit von 40-kHz Rufen in der zweiten Isolation deutlich über der in der ersten Isolation. Kontrolltiere, welche lediglich Kontakt zu einem Geschwistertier hatten, zeigen diesen Anstieg nicht (Hofer, 1996; Muller et al., 2005; 2008). Dieses Phänomen wird unter Heranziehung von Kosten-Nutzen-Modellen erklärt. Da das Vokalisationsverhalten des Jungtiers mutmaßlich mit einem erhöhten Verbrauch von Energiereserven einhergeht und außerdem die Wahrscheinlichkeit erhöht von einem Freßfeind entdeckt zu werden, erscheint ein konstantes, durch soziale Faktoren nicht zu modulierendes Rufverhalten den Überlebenschancen des Jungtieres abträglich. Vielmehr sollte ein Rufverhalten, das auf die wahrscheinliche Nähe der Mutter abgestimmt ist, die Überlebenschancen des Jungtieres erhöhen. Ein vor kurzem aufgetretener Kontakt mit der Mutter kann in diesem Sinne als Hinweisreiz für die Nähe der Mutter verstanden werden und folglich für eine hohe Wahrscheinlichkeit, dass die Mutter die Rufe des Jungtieres wahrnehmen kann und diese Eintragverhalten zur Folge haben (Hofer, 1996). Abgesehen von diesen relativen kurzzeitigen Effekten der Mutter auf das Rufverhalten des Jungtiers liegen jedoch wenige Hinweise dafür vor, dass die Mutter langanhaltend das Rufverhalten ihrer Nachkommen beeinflussen könnte. Lediglich Darnaudery et al. (2004) konnte im Rahmen einer Adoptionsstudie Hinweise dafür erbringen, dass ein ausgeprägtes Pflegeverhalten mit einem reduziertem Vokalisationsverhalten der Jungtiere einhergeht. Maternale Fürsorge reduziert bekanntermaßen angst-ähnliches Verhalten der Nachkommen (Caldji et al., 1998; Francis et al. 1999; Menard et al., 2004; Menard & Hakvoort, 2007; Zhang et al., 2005).

Ultraschallvokalisationen bei der Ratte
- 22-kHz Vokalisationen

22-kHz Vokalisationen und affektiver Zustand

22-kHz Vokalisationen werden von juvenilen und adulten Ratten in einer Vielzahl aversiver Situationen emittiert, so etwa während innerartlicher Kämpfe (Kaltwasser, 1990a; Kroes et al. et al., 2007; Lehman & Adams, 1977; Lore et al, 1976; Sales 1972a; Thomas et al., 1983), sozialer Isolation (Francis, 1977), Drogenentzug (Barros & Miczek, 1996), Handhabung durch einen Menschen (Brudzynski & Ociepa, 1992), nach Verabreichung eines elektrischen Schlags (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005) oder lauten Geräuschen (Kaltwasser, 1990b; 1991), sowie bei Konfrontation mit einem Fressfeind (Blanchard et al., 1990; 1991; 1992; Shepherd et al., 1992; zur Übersicht siehe: Litvin et al., 2007). Diese Vokalisationen können aber interessanterweise nicht allein in der aktuellen Bedrohungssituation beobachtet werden, sondern auch in Antizipation einer solchen. Im Rahmen von Studien zur Furchtkonditionierung [*fear conditioning*] konnte beispielsweise gezeigt werden, dass 22-kHz Vokalisationen nicht allein am Tag der Konditionierung auftreten, an welchem Ton (konditionierter Stimulus; CS) und elektrischer Schlag (unkonditionierter Stimulus; US) gepaart dargeboten wurden, sondern auch in der

anschließenden Testphase, allein in Reaktion auf den CS (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005).

Ausgehend von diesen Beobachtungen wurde postuliert, dass 22-kHz Vokalisationen einen negativen affektiven Zustand, wie etwa Angst reflektieren (Jelen et al., 2003). Tatsächlich konnte gezeigt werden, dass die Auftretenshäufigkeit der 22-kHz Rufe abhängig ist von der Aversivität der Situation (Wöhr et al., 2005). So vokalisierten Tiere mit zunehmender Stärke des in der Furchtkonditionierung verwandten elektrischen Schlags mehr. Doch nicht alle Tiere sandten 22-kHz-Rufe aus. Auch konnte beobachtet werden, dass die Dauer der Verhaltensstarre und die Emission von 22-kHz-Rufen positiv korrelierten, d.h. dass Tiere, die auf der Ebene des sichtbaren Verhaltens mehr furcht-ähnliches Verhalten zeigten auch mehr vokalisierten, als jene, die nicht so lange in Verhaltensstarre verharrten (Burgdorf et al., im Druck; Choi & Brown, 2003; Wöhr et al., 2005). Beide Beobachtungen legen nahe, dass neben den situativen Charakteristika einer Bedrohung, auch Merkmale des Tieres selbst einen Einfluss darauf haben, ob und wie ausgeprägt es 22-kHz-Rufe aussendet.

Interessanterweise ist seit langem bekannt, dass sich Tiere in ihrer individuellen Ausprägung der Disposition Ängstlichkeit unterscheiden (Borta et al., 2006; Caldji et al., 1998; Francis et al., 1999; Ho et al., 2002; 2004; Menard et al., 2004; Menard & Hakvoort, 2007; Pawlak et al., 2003; Ray & Hansen, 2005; Schwarting & Pawlak, 2004; Schwarting et al., 1998; Zhang et al., 2005). Tatsächlich konnte gezeigt werden, dass Tiere, welche vor Durchführung der Furchtkonditionierung anhand ihres unkonditionierten angst-ähnlichen Verhaltens auf dem erhöhten Pluslabyrinth als niedrig- beziehungsweise als hoch-ängstlich eingestuft wurden, sich in ihrem Vokalisationsverhalten während der Furchtkonditionierung unterscheiden (Borta et al., 2006). Als hoch-ängstlich eingestufte Tiere vokalisierten mehr als jene Tiere, welche als niedrig-ängstlich eingestuft wurden - ein Unterschied, welcher nicht auf eine unterschiedliche Schmerzsensitivität der Tiere zurückgeführt werden konnte (Borta et al., 2006). Diese Beobachtung steht in Übereinstimmung zu Studien mit Tieren, die in Abhängigkeit ihres juvenilen oder adulten angst-ähnlichen Verhaltens gezüchtet wurden. Tiere der hoch-ängstlichen Linien zeigen mehr 22-kHz Rufe in aversiven Situationen als Tiere der niedrig-ängstlichen Linien und zwar unabhängig davon, ob die Tiere gemäß der isolations-induzierten Jungtiervokalisationen (Brunelli, 2005), dem angst-ähnlichen Verhalten auf dem erhöhten Pluslabyrinth (Frank et al., 2006) oder der lokomotorischen Aktivität in aversiven Situationen gezüchtet wurden (Naito et al., 2001).

Auch konnte gezeigt werden, dass anxiolytische Substanzen das Vokalisationsverhalten reduzieren können (Jelen et al., 2003; zur Übersicht siehe: Sanchez,

2003). Hier sind insbesondere die Benzodiazepine, wie etwa Diazepam, zu nennen. Ferner erwiesen sich Serotoninwiederaufnahmehemmer und andere Substanzen, welche die serotonerge Neurotransmission erhöhen, als rufreduzierend. Studien, bei welchen der muscarinerge Agonist Carbachol intrazerebral verabreicht wurde, legen nahe, dass eine Aktivierung des cholinergen Systems hingegen 22-kHz Rufe induzieren kann (Bihari et al., 2003; Brudzynski, 1994; Brudzynski & Barnabi, 1996; Brudzynski & Bihari, 1990; zur Übersicht siehe: Brudzynski, 2007).

Ergänzt werden diese neuropharmakologische Studien durch Experimente, die eine Beteiligung von Amygdala und zentralem Höhlengrau an der Produktion von 22-kHz Rufen nachweisen konnten (Choi & Brown, 2003; Depaulis et al., 1992, Kroes et al., 2007). Amygdala und zentrales Höhlengrau sind bekanntermaßen Hirnregionen, die im Zusammenhang mit der Regulation von Angst und Furcht stehen (LeDoux, 2000). Im Rahmen einer Furchtkonditionierungsstudie konnte beispielsweise gezeigt werden, dass eine Läsion der Amygdala vor Durchführung der Konditionierung zu einem Ausbleiben von 22-kHz Rufen in Reaktion auf einen CS führte. Da jedoch 22-kHz Rufe in Reaktion auf einen US weiterhin auftraten, muss gefolgert werden, dass eine intakte Amygdala nicht notwendig ist für die Emission von 22-kHz Rufen (Choi & Brown, 2003). Die Produktion der Rufe selbst scheint vielmehr vom zentralen Höhlengrau abhängig zu sein. So zeigten Depaulis et al. (1992), dass eine pharmakologische Erregung des zentralen Höhlengraus zur Emission von 22-kHz Rufen führt. Ergänzend hierzu konnte beobachtet werden, dass agonistische Auseinandersetzungen beim unterlegenen Tier neben der Produktion von 22-kHz Rufen zu einer Veränderung der Genexpression im zentralen Höhlengrau führen. Bemerkenswerterweise handelt es sich hierbei um Gene, welche an der Regulation der cholinergen Neurotransmission beteiligt sind (Kroes et al., 2007).

Signalfunktion von 22-kHz Vokalisationen

Hinsichtlich der kommunikativen Bedeutung von 22-kHz Vokalisationen liegen widersprüchliche Befunde vor. So gibt es Hinweise, dass diese Rufe eine Alarmfunktion für Artgenossen besitzen. Es konnte gezeigt werden, dass Ratten bei Konfrontation mit einem Fressfeind, wie etwa einer Katze, vor allem dann 22-kHz-Rufe aussenden, wenn Artgenossen anwesend sind (Blanchard et al., 1991; zur Übersicht siehe: Litvin et al., 2007). In Übereinstimmung mit einer potentiellen Alarmfunktion dieser Rufe, konnte in mehreren Studien gezeigt werden, dass Tiere in Reaktion auf die Präsentation von künstlichen 20-kHz Sinustönen Verhaltensstarre zeigen (Commissaris et al., 2000; Neophytou et al., 2000) oder

fliehen (Beckett et al., 1996; 1997; Commissaris et al., 1998; 2000; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Voits et al., 1999). Diese Verhaltensreaktionen werden von einer erhöhten Aktivität in Amygdala und zentralem Höhlengrau begleitet (Beckett et al., 1997; Neophytou et al., 2000). Interessanterweise konnte ferner gezeigt werden, dass diese Verhaltensreaktionen durch anxiolytische Substanzen abgeschwächt werden können (Beckett et al., 1996; Nicolas et al., 2007). Es muss jedoch angemerkt werden, dass in anderen Studien unter Verwendung natürlicher 22-kHz Vokalisationen nur eine schwache (Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991) oder überhaupt keine (Bang et al., im Druck; Lindquist et al., 2004; Tankhiwale et al., 2007) Änderungen des Verhaltens zu beobachten war. Die zu beobachtenden schwachen Effekte waren zudem nicht spezifisch für natürliche 22-kHz Vokalisationen, sondern waren auch in Folge der Darbietung von artifiziellen Ultraschallsignalen mit ähnlichen Frequenzen zu beobachten (Endres et al., 2007; Sales, 1991).

Sales (1972a) hingegen stellte die Hypothese auf, dass diese Rufe im innerartlichen Kampf durch Signalisieren der Unterlegenheit dazu dienen, das überlegene Tier von weiteren Angriffen abzuhalten. Sie konnte beobachten, dass Tiere, welche in Konfrontationen 22-kHz Vokalisationen ausstießen, in deren Folge kaum noch angegriffen wurden, wohingegen Tiere, die keine 22-kHz Vokalisationen emittierten, mehr und über einen längeren Zeitraum angegriffen wurden. Diese Beobachtung konnte in folgenden Studien bestätigt werden (Lehman & Adams, 1977; Lore et al, 1976). Auch Studien, in welchen eine Inhibition lokomotorischer Aktivität in Folge des Präsentierens von 22-kHz Vokalisationen oder 20-kHz Sinustönen beobachtet wurde, sind vereinbar mit dieser Hypothese (Brudzynski & Chiu, 1995; Burman et al., 2007; Commissaris et al., 2000; Endres et al., 2007; Neophytou et al., 2000; Sales, 1991). Für die Annahme, dass 22-kHz-Vokalisationen eine funktionale Bedeutung haben und zur Stabilisierung des Sozialverbandes beitragen, sprechen auch Befunde bezüglich des Einflusses von Haltungsbedingung auf die Entwicklung der Emission von 22-kHz Vokalisationen. Inagaki et al. (2004) zeigten, dass Ratten, welche nach der Trennung von der Mutter über sechs Monate hinweg allein gehalten wurden, in aversiven Situationen kaum 22-kHz Rufe ausstießen, wohingegen in Paaren aufgewachsene Ratten in der gleichen Situation häufig vokalisierten. Die Beobachtung, dass isoliertes Aufwachsen die Emission von 22-kHz Rufen reduziert, wurde seither mehrfach bestätigt (Nunes Mamede Rosa et al., 2005; Tomazini et al., 2006). Für ein erfolgreiches Erlernen des Gebrauchs der 22-kHz Vokalisationen erscheinen soziale Interaktionen daher bedeutsam zu sein. Weiterhin konnten Inagaki et al. (2004) zeigen, dass auch die Dominanzstruktur eine Einfluss auf die

Emission von 22-kHz Rufen hat. Die unterlegenen Ratten vokalisierten signifikant häufiger als die dominanten Ratten. Andererseits konnte in einer Untersuchung, bei der gezielt das unterlegene Tier operativ devokalisiert wurde, kein Anstieg von Angriffen beobachtet werden (Thomas et al., 1983). In einem weiteren Versuch wurde ferner gezeigt, dass auch der experimentell erzeugte Verlust des Hörvermögens des überlegenen Tieres und somit das Unvermögen Unterlegenheitssignale des Kontrahenten zu hören, keinen Einfluss auf das Verhalten während der Auseinandersetzung hatte (Thomas et al., 1983).

Schließlich besteht noch die Hypothese, dass 22-kHz Vokalisationen dazu dienen, Fressfeinde von einem Angriff abzuhalten (Endres et al., 2007). Es ist bekannt, dass eine Vielzahl von Tieren Signale aussenden, die dazu dienen dem Angreifer zu signalisieren, dass er entdeckt wurde und ein Angriff daher wenig erfolgsversprechend ist (zur Übersicht siehe: Shelley & Blumstein, 2005). Tatsächlich emittieren Ratten 22-kHz Vokalisationen bei Konfrontation mit einer Katze (Blanchard et al., 1990; 1991; 1992; Shepherd et al., 1992; zur Übersicht siehe: Litvin et al., 2007). Ob diese Rufe jedoch tatsächlich das Verhalten des Fressfeindes verändern können wurde bisher nicht experimentell geprüft.

Ultraschallvokalisationen bei der Ratte
- 50-kHz Vokalisationen

50-kHz Vokalisationen und affektiver Zustand

Im Gegensatz zu 22-kHz Vokalisationen emittieren juvenile und adulte Ratten Rufe im Bereich von 50 kHz typischerweise in Situationen, in denen Belohnungen erhalten werden oder antizipierbar sind und Annäherungsverhalten adaptiv ist. Im natürlichen Kontext treten diese Rufe vor allem in Antizipation oder während des Spiels junger Ratten auf (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998), sowie während sozialer Exploration [*social investigation*] (Sales, 1972a) und Paarung adulter Tiere (Barfield et al., 1979; Bialy et al., 2000; Burgdorf et al., im Druck; Geyer & Barfield, 1978; McGinnis & Vakulenko, 2003; McIntosh et al., 1978; Sales, 1972b; White & Barfield, 1990; White et al., 1990).

Im Labor lassen sich die 50-kHz Rufe auch durch Kitzeln der Ratte durch den Versuchsleiter auslösen (Burgdorf & Panksepp, 2001; Burgdorf et al., im Druck; Mällo et al., 2007; Panksepp & Burgdorf, 1999; 2000; 2003; Schwarting et al., 2007). Hierbei wird das juvenile Spiel der Ratte nachgestellt, indem das Tier mit der Hand verfolgt, umgeworfen und in den Nacken gekniffen wird. Außerdem treten die Rufe nach Gabe von suchtgefährdenden Substanzen auf, wie beispielsweise Amphetamin (Burgdorf et al., 2001; Knutson et al., 1999;

Thompson et al., 2006; Wintink & Brudzynski, 2001). Diese Tatsache und die Beobachtung, dass die elektrische Stimulation dopaminerger Bahnen im Gehirn ebenfalls zu 50-kHz Vokalisationen führt (Burgdorf et al., 2000; 2007), verweist auf die zentrale Rolle des dopaminergen Systems für die Produktion dieser Vokalisationen. Tatsächlich konnte gezeigt werden, dass nach elektrolytischer oder pharmakologischer Ausschaltung des ventralen Tegmentums 50-kHz Vokalisationen deutlich seltener auftreten, wie auch nach Gabe des D1/D2-Dopamin-Antagonisten Flupenthixol (Burgdorf et al., 2007).

Es wird daher angenommen, dass dieser Ruftyp einen positiven affektiven Zustand, wie Freude, widerspiegelt und eine archaische Form menschlichen Lachens darstellt (Panksepp & Burgdorf, 2003). In Übereinstimmung mit dieser Annahme konnte gezeigt werden, dass die Emission von 50-kHz Rufen und der anhand des Annäherungsverhaltens erfasste Anreizcharakter des Kitzelns positiv korreliert sind (Burgdorf & Panksepp, 2001; Panksepp & Burgdorf, 1999; 2003). Ferner konnte gezeigt werden, dass aversive Stimuli, wie beispielsweise helles Licht (Knutson et al., 1998; Panksepp & Burgdorf, 1999), der Geruch einer Katze (Panksepp & Burgdorf, 1999) oder ein Hinweisreiz auf einen elektrischen Schlag (Burgdorf et al., 2000), die Rate der 50-kHz Rufe dramatisch absenken kann. Außerdem reduzieren aversive Substanzen, wie beispielsweise Lithiumchlorid die Emission von 50-kHz Rufen (Burgdorf et al., 2001). Widersprüchliche Befunde, wie etwa die Tatsache, dass Tiere bei innerartlichen Kämpfen 50-kHz Vokalisationen emittieren (Burgdorf et al., im Druck; Haney & Miczek, 1994; Sales, 1972a; Thomas et al., 1983; Tornatzky & Miczek, 1994; 1995; Vivian & Miczek, 1993a; 1993b), wird als Freude der Überlegenheit eines der beiden am Kampf beteiligten Tiere interpretiert (Panksepp & Burgdorf, 2003). Tatsächlich ist die Verhaltensstarre, die das unterlegene Tier zeigt, negativ mit der Emission von 50-kHz Rufen korreliert (Burgdorf et al., im Druck).

Im Widerspruch zur Hypothese, dass diese Rufe einen positiven affektiven Zustand reflektieren, steht aber der Befund, dass viele Tiere in Situationen, die keinen appetitiven Charakter aufweisen, 50-kHz Vokalisationen aussenden. So konnte beispielsweise beobachtet werden, dass zahlreiche Kontrolltiere, welche im Gegensatz zu den Experimentaltieren keinen Sozialkontakt (Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003) oder eine Injektion von Amphetamin erwarten konnten (Knutson et al., 1999; Thompson et al., 2006; Wintink & Brudzynski, 2001), 50-kHz Vokalisationen aussenden. Ausgehend von diesen Beobachtungen wurde der postulierte Zusammenhang zwischen 50-kHz Vokalisationen und positivem affektiven Zustand spezifiziert. So wird nunmehr zwischen zwei Typen von 50-kHz Rufen differenziert. Mit einem positiven affektiven Zustand sollen allein frequenzmodulierte 50-kHz

Rufe, nicht aber flache 50-kHz Rufe, in Zusammenhang stehen (Burgdorf & Panksepp, 2006; Burgdorf et al., 2007; im Druck). Tatsächlich treten während des Kitzelns der Ratten fast ausschließlich frequenzmodulierte Rufe auf (Burgdorf et al., im Druck). Ihre Auftretenshäufigkeit, nicht aber die der flachen 50-kHz Rufe, lässt sich durch eine dem Kitzeln vorangehende soziale Isolation der Tiere noch erhöhen (Burgdorf et al., im Druck). Bei Paarung und Spiel sind lediglich frequenzmodulierte 50-kHz Rufe mit Annäherungsverhaltensweisen, wie Verfolgen des Artgenossen, korreliert (Burgdorf, im Druck). Schließlich wirkt sich eine Ausschaltung dopaminerger Neurotransmission allein auf die Produktion von frequenzmodulierten 50-kHz Rufe hemmend aus (Burgdorf et al., 2007).

Signalfunktion von 50-kHz Vokalisationen

Über die kommunikative Bedeutung von 50-kHz Rufen ist bisher nur wenig bekannt und die Befundlage ist widersprüchlich. Ähnlich wie bei den 22-kHz Vokalisationen hat die Tatsache, dass diese Vokalisationen während innerartlichen Kämpfen auftreten (Burgdorf et al., im Druck; Haney & Miczek, 1994; Sales, 1972a; Thomas et al., 1983; Tornatzky & Miczek, 1994; 1995; Vivian & Miczek, 1993a; 1993b) dazu geführt, dass eine den Kontrahenten beschwichtigende Funktion angenommen wurde (Brudzynski & Pniak, 2002; Tornatzky & Miczek, 1994; 1995). In Übereinstimmung mit dieser Hypothese konnte mittels Devokalisationsstudien tatsächlich beobachtet werden, dass vor allem jenes Tier, welches in ein fremdes Territorium eindringt, 50-kHz Vokalisationen aussendet (Takahashi et al., 1983; Thomas et al., 1983). Auch konnte gezeigt werden, dass sich die Auftretenshäufigkeit der in Antizipation eines Kampfes ausgesandten 50-kHz Vokalisationen mittels Anxiolytika, wie Diazepam, reduzieren lassen (Tornatzky & Miczek, 1995). Allerdings konnte in einer Studie, bei welcher der Eindringling devokalisiert wurde, keine Veränderungen im aggressiven Verhalten des Kontrahenten beobachtet werden (Thomas et al., 1983).

Gemäß einer anderen Hypothese wird angenommen, dass die Produktion von 50-kHz Rufen dem Paarungserfolg dient (Geyer et al., 1978; McIntosh et al., 1978; White & Barfield, 1987; 1989; 1990). So konnte gezeigt werden, dass Weibchen mehr Paarungsverhalten zeigen, wenn sie vor der Paarung 50-kHz Rufe gehört hatten (Geyer et al., 1978). Auch wirkten sich 50-kHz Rufe während der Paarung positiv auf das Paarungsverhalten des Weibchens aus (McIntosh et al., 1978; White & Barfield, 1990). Eine weitere Studie zeigte, dass das Weibchen auf das Abspielen männlicher 50-kHz Rufe selbst mit 50-kHz Vokalisationen antwortet (White et al., 1993). Eine Devokalisation des Weibchens führt hingegen zu einer als kompensatorisch beschriebenen Steigerung ihres Paarungsverhaltens

(White & Barfield, 1987; 1989). Zusammenfassend kann daher geurteilt werden, dass 50-kHz Rufe eine funktionale Bedeutung für das Paarungsverhalten haben. Die Tatsache jedoch, dass diese Rufe in ähnlich starken Maße auch in nicht-sexuellen Situationen, wie Spiel (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998) und Kampf (Burgdorf et al., im Druck; Haney & Miczek, 1994; Sales, 1972a; Thomas et al., 1983; Tornatzky & Miczek, 1994; 1995; Vivian & Miczek, 1993a; 1993b), auftreten, zeigt, dass die Funktion von 50-kHz Rufen umfassender sein muss.

Im nicht-sexuellen Kontext wurden jedoch kaum Studien zur funktionalen Relevanz von 50-kHz Rufen durchgeführt. Auf eine funktionale Rolle verweist die Beobachtung, dass die Ausschaltung des Hörvermögens das Spielverhalten junger Ratten beeinflusst (Siviy & Panksepp, 1987). Außerdem wurde beobachtet, dass Ratten mehr Zeit mit Artgenossen verbringen, die viel vokalisieren, als mit solchen, die wenig 50-kHz Rufe aussenden (Panksepp et al., 2002). Möglicherweise werden die 50-kHz Vokalisationen also ausgesandt, um Kontakt mit Artgenossen herzustellen. Dafür sprechen auch die Beobachtungen, dass zahlreiche Kontrolltiere in non-appetitiven Situationen 50-kHz Rufe produzieren (Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Knutson et al., 1999; Thompson et al., 2006; Wintink & Brudzynski, 2001). Auch der Befund, dass allein das Versetzen von Artgenossen in einen neuen Käfig zu 50-kHz Rufen führt (Schwarting et al., 2007), könnte damit im Zusammenhang stehen. Ausgeschlossen werden kann bisher lediglich, dass es um eine reine Reaktion auf neue Reize handelt, da dieses Vokalisationsverhalten bei wiederholter Testung über mehrere Tage hinweg nicht habituiert (Schwarting et al., 2007). Allerdings stützten Untersuchungen, bei denen 50-kHz Rufe künstlich dargeboten wurden, die Annahme, dass 50-kHz Vokalisationen dazu dienen, Sozialkontakt aufzunehmen, nicht. Entgegen der hieraus abzuleitenden Erwartung, dass sich Tiere 50-kHz Rufen annähern, wurde keine Verhaltensänderung durch das Abspielen von 50-kHz Rufen beobachtet (Burman et al., 2007; Endres et al., 2007).

Ultraschallvokalisationen bei der Maus
- 60-kHz Vokalisationen

60-kHz Vokalisationen und affektiver Zustand

Wie auch die junge Ratte emittiert die junge Maus Vokalisationen in potentiell lebensbedrohlichen Situationen, wie etwa nach einer Trennung von Mutter und Geschwistern (Zippelius & Schleidt, 1956). Diese Vokalisationen haben typischerweise eine Frequenz von etwa 60 kHz (Hashimoto et al., 2001). In Anlehnung an die Befunde bei der Ratte wird davon ausgegangen, dass diese Rufe einen negativen affektiven Zustand reflektieren (Branchi et al., 2001). Tatsächlich wirken sich auch bei der Maus Anxiolytika rufmindernd aus (Benton & Nastiti, 1988; Krömer et al., 2005). Auch zeigen Jungtiere einer Linie, die gemäß einem stark ausgeprägtem angst-ähnlichen Verhalten auf dem erhöhten Pluslabyrinth gezüchtet wurden, mehr Rufe in Isolation als Tiere einer Linie, die gemäß einem schwach ausgeprägtem angst-ähnlichen Verhalten auf dem erhöhten Pluslabyrinth gezüchtet wurden (Krömer et al., 2005).

Aufgrund der Tatsache, dass eine große Zahl transgener Tiere hergestellt wurde, ist über die genetischen Grundlagen des Vokalisationsverhaltens bei der jungen Maus deutlich mehr bekannt als bei der Ratte, wo die Bedeutung genetischer Faktoren für die Produktion von Ultraschallvokalisationen bislang allein durch selektive Züchtung (Brunelli & Hofer, 2007; Brunelli, 2005; Brunelli et al., 1997; 2001; 2002; Hofer et al., 2001; Shair et al., 2000;

Zimmerberg et al., 2005) und Adoptionsstudien (Brunelli et al., 2001; Graham & Letz, 1979) demonstriert werden konnte. Auf die große Bedeutung genetischer Faktoren bei der Maus wiesen die Ergebnisse einer frühen Studie von Bell et al. (1972) hin, in welcher gezeigt werden konnte, dass die Auftretenshäufigkeit von Ultraschallvokalisationen bei der Maus in starkem Maße vom Stamm des Tieres abhängt. Ähnliche Ergebnisse wurden seitdem in verschiedenen Untersuchungen erzielt (Cohen-Salmon et al., 1985; Hennessy et al., 1980; Robinson & D'Udine, 1982; Sales & Smith, 1978). Weiterführende genetische Analysen konnten Belege dafür liefern, dass Rufanzahl und alle Rufmerkmale von einem multiplen genetischen Hintergrund abhängig sind (Hahn et al., 1987; 1997; 1998; Hahn & Schanz, 2002; Roubertoux et al., 1996; Thornton et al., 2005).

Die Beteiligung zahlreicher Gene an der Produktion von Ultraschallvokalisationen konnte auch anhand verschiedener Knock-Out und Knock-In Tiere beobachtet werde. So ist beispielsweise das Gen Foxp2 von enormer Bedeutung für die Rufproduktion der Maus (Fujita et al., 2008; Groszer et al., 2008; Shu et al., 2005), welches interessanterweise beim Menschen an der Sprachproduktion beteiligt ist (Lai et al., 2001). Neben Genen, die vermutlich für den Aufbau des Vokalisationsapparates von Bedeutung sind, spielen Gene, die am Aufbau von Rezeptoren für verschiedene Neurotransmitter und Neuropeptide beteiligt sind, eine wichtige Rolle. In Übereinstimung mit pharmakologischen Studien (Benton & Nastiti, 1988; Krömer et al., 2005) zeigten Untersuchungen an Knock-Out Tieren, dass eine ganze Reihe unterschiedlicher Neurotransmittersysteme in der Produktion der Rufe involviert sind. Zuvorderst ist hier Serotonin zu nennen (Brunner et al., 1999; El-Khodor et al., 2004; Weller et al., 2003), dessen Beteiligung wahrscheinlich auf seine vermutete Rolle bei der Regulation von Angst und Furcht (Handley & Blane, 1993) zurückzuführen ist. Ferner wird das Vokalisationsverhalten der Jungtiere auch durch Oxytozin (Winslow et al., 2000) und Vasopressin (Scattoni et al., 2007) beeinflusst. Diese Neuropeptide sind bekanntermaßen entscheidend für den Aufbau sozialer Bindungen (zur Übersicht siehe: Bartz & Hollander, 2006; Lim & Young, 2006; Insel & Young, 2001). Schließlich sind noch die Opioide zu nennen, deren Einfluss ebenfalls über eine Modulation sozialen Interesses erklärt wird. Ein Fehlen des mu-opioid-Rezeptors führt beinahe zum vollständigen Ausbleiben isolations-induzierter Ultraschallvokalisation (Moles et al., 2004).

Dennoch zeigen Verhaltensuntersuchungen, dass auch Umweltfaktoren die Ultraschallvokalisation der Mäuse beeinflussen können. Werden Jungtiere beispielsweise von tauben Müttern aufgezogen so emittieren diese Tiere weniger 60-kHz Rufe als Kontrolltiere (D'Amato & Populin, 1987). Auch die Potentierung des Rufverhaltens durch einen kurzen

Kontakt mit der Mutter konnte bei Mäusen beobachtet werden (Scattoni et al., 2007). Dass jedoch auch die beobachteten Stammesunterschiede nicht allein auf genetische Faktoren zurückzuführen sind, verdeutlichten Ergebnisse einer Adoptionsstudie von D'Amato et al. (2005). Es konnte gezeigt werden, dass Jungtiere unabhängig von ihrem genetischen Hintergrund in Isolation weniger 60-kHz Rufe produzierten wenn sie von den stark maternal responsiven Müttern des Stamms C57BL/6 aufgezogen wurden und nicht von den wenig maternal responsiven Müttern des Stamms BALB/c.

Signalfunktion von 60-kHz Vokalisationen

Nachdem Zippelius und Schleidt (1956) beobachtet hatten, dass junge Mäuse, die nach Trennung von Mutter und Geschwister Ultraschallvokalisationen aussenden, von ihrer Mutter in das Nest eingetragen wurden, lieferte Sewell (1970) den experimentellen Nachweis, dass die isolations-induzierten Ultraschallrufe und nicht ein anderer mit der Isolation einhergehender Stimulus verantwortlich ist für die Induktion des maternalen Eintrageverhaltens. Sie zeigte, dass laktierende Waldmausweibchen in Reaktion auf das Abspielen von isolations-induzierten Ultraschallrufen vom Tonband mit einem Verlassen ihres Nestes reagierten und das umliegende Gelände explorierten. Diese Exploration war meist auf den Lautsprecher hin ausgerichtet. Kontrollsignale, wie zum Beispiel ein künstlicher 45-kHz Ton, erweisen sich hingegen als ungeeignet. Interessanterweise beobachtete Sewell (1970) diese Verhaltensreaktionen, ohne dass zusätzlich zu den akutischen Stimuli Gerüche eines Jungtieres verwendet wurden.

Unter Verwendung von artifiziellem Stimulusmaterial konnte in darauf folgenden Studien bestimmt werden, welche Rufmerkmale entscheidend für die Induktion des Eintrageverhaltens sind. Demnach können alle Signale im Hörbereich der Maus mit einer Frequenz von über 35 kHz und einer Mindestdauer von 25 ms Eintrageverhalten induzieren, sofern in den benachbarten Frequenzbändern eine um mindestens 20 dB niedrigere Schallamplitude besteht (Ehret, 1992; Ehret & Haack, 1981; 1982).

Ultraschallvokalisationen bei der Maus
- 70-kHz Vokalisationen

70-kHz Vokalisationen und affektiver Zustand

Juvenile und adulte Mäuse zeigen keine Alarmrufe vom Typ der 22-kHz Rufe der Ratte – ein Umstand, welcher auf die Tatsache zurückgeführt wird, dass Mäuse im Gegensatz zu Ratten nicht in strukturierten Sozialverbänden leben, bei welchen es bestimmten Mitgliedern obliegt, Artgenossen vor Gefahren zu warnen (Blanchard et al., 2001). Es konnte bei ihnen jedoch ein hochfrequenter „Gesang“ beobachtet werden, welcher eine große Ähnlichkeit zu den 50-kHz Rufen bei der Ratte aufweist (Holy & Guo, 2005). Tatsächlich treten die 70-kHz Vokalisationen der juvenilen und adulten Mäuse in ähnlichen Situationen auf wie die 50-kHz Rufe der Ratte - insbesondere während der Paarung (Sales, 1972b; Nyby, 1983; Wang et al., 2008; White et al., 1998). Bereits der Geruch eines Weibchens ist ausreichend, um Rufverhalten beim Männchen auszulösen (Holy & Guo, 2005; Nyby et al., 1977; Wang et al., 2008).

Darüber hinaus konnten auch bei gleichgeschlechtlichen Interaktionen 70-kHz Rufe beobachtet werden (D’Amato & Moles, 2001; Maggio & Whitney, 1985; Moles & D’Amato, 2000; Sewell, 1970; Panksepp et al., 2007). Diese Rufe treten vor allem zu Beginn der Interaktion auf (D’Amato & Moles, 2001). In Übereinstimmung mit der Ratte konnte eine

prominente Rolle des dopaminergen Systems nachgewiesen werden. So lassen sich 70-kHz Vokalisationen in hohem Maße durch Verabreichung von Amphetamin stimulieren (Wang et al., 2008). Es wurde daher angenommen, dass auch diese Vokalisationen einen positiven affektiven Zustand reflektieren (Wang et al., 2008).

Signalfunktion von 70-kHz Vokalisationen

Wiederum basierend auf den Situationen, in welchen die 70-kHz Rufe auftreten, wurde zum einen vermutet, dass diese das Paarungsverhalten erleichtern (Nyby & Whitney, 1978), zum anderen, dass sie allgemein dazu dienen, Sozialkontakt herzustellen (Moles & D'Amato, 2000). Für die erstere Annahme spricht, dass Weibchen sich mehr in der Nähe eines vokalisierenden als eines devokalisierten Männchens aufhalten (Pomerantz et al., 1983). Bemerkenswerterweise war diese Präferenz der Weibchen nicht nach Ovariektomie zu beobachten, was auf die Rolle von Geschlechtshormonen hinweist (Pomerantz et al., 1983). Auch hier spricht jedoch die Tatsache, dass 70-kHz Vokalisationen ebenfalls in nicht-sexuellen Kontexten auftreten (D'Amato & Moles, 2001; Maggio & Whitney, 1985; Moles & D'Amato, 2000; Sewell, 1970; Panksepp et al., 2007) gegen eine begrenzte Funktionalität dieser Rufe.

Für die Annahme, dass die 70-kHz Rufe vielmehr allgemein der Herstellung von Sozialkontakt dienen, spricht eine Vielzahl von Befunden. So ist auffällig, dass die Rufe parallel zur sozialen Exploration auftreten (Sales, 1972a; Maggio & Whitney, 1985). Beispielsweise korreliert die Zeit, welche die Tiere mit dem Beschnuppern der Partners verbringen, positiv mit den emittierten 70-kHz Rufen (Moles et al., 2007; Panksepp et al., 2007). Außerdem konnte gezeigt werden, dass ihre Anzahl ansteigt, wenn der Partner einen für das Überleben dienlichen Hinweisreiz, wie etwa den Geruch nach einem neuen Futter, mit sich führt (Moles & D'Amato, 2000). Schließlich besteht auch ein Zusammenhang zwischen der Bekanntheit des Partners und der Anzahl der aufgetretenen Rufe. Ist der Partner unbekannt, so werden viele 70-kHz Rufe produziert. Ist der Partner bekannt, so sinkt mit der Zeit, welche das Tier mit sozialer Investigation verbringt, auch die Anzahl der 70-kHz Rufe (Moles et al., 2007). Es erscheint daher möglich, dass die 70-kHz Rufe der adulten Maus ein Maß zur Erfassung des sozialen Interesses des Tieres darstellen (Moles et al., 2007).

FRAGESTELLUNG UND HYPOTHESEN

Der aktuelle Wissenstand zur sozialen Funktion von Ultraschallvokalisationen ist gekennzeichnet durch Lücken und widersprüchliche Befunde. Dies könnte unter anderem auf die in einigen der durchgeführten Untersuchungen verwendeten Methoden, wie zum Beispiel eine aus heutiger Sicht unzureichende Technik zur Präsentation von Ultraschallsignalen, oder auf einen zu stark eingeschränkten Forschungsbereich, wie im Falle der Bedeutung von 50- und 70-kHz Vokalisationen für das Sexualverhalten, zurückzuführen sein. Die Durchführung weiterer Experimente unter Verwendung modernster Technik in einem breiteren Forschungsfeld erscheint notwendig. Ziel der vorgelegten Studien war es daher unter Berücksichtigung dieser Prämissen einen Beitrag hierzu zu leisten, um die Möglichkeit zu gewinnen auf einer breiteren wissenschaftlichen Basis zu prüfen, inwiefern es sich bei den Ultraschallvokalisationen um ein kommunikatives Signal des motivational-affektiven Zustands handelt.

Hierzu wurde zum einen untersucht, welche Bedeutung soziale Faktoren, wie maternale Fürsorge oder An- beziehungsweise Abwesenheit eines Artgenossen, für den Sender, das heißt für die Produktion von Rufen, haben. Gemäß der Annahme, dass 40-kHz und 22-kHz Vokalisationen der Ratte einen negativen affektiven Zustand reflektieren (Hofer,

1996; Jelen et al., 2003), wurde erwartet, dass diese im Zuge einer umfassenden Abnahme angst-ähnlichen Verhaltens in Folge ausgeprägter maternaler Fürsorge (Caldji et al., 1998; Francis et al. 1999; Menard et al., 2004; Menard & Hakvoort, 2007; Zhang et al., 2005) ebenfalls seltener auftreten (Studie I). Um diese angenommene Kausalität zu prüfen, wurde ferner unter Verwendung von Mäusen eine Emryotransferstudie durchgeführt, so dass die Bedeutung genetischer als auch epigenetischer Faktoren für die Produktion von 60-kHz Rufen gesondert erfasst werden konnte (Studie II). Hinsichtlich der 22-kHz Vokalisationen der Ratte wurde weiterhin erwartet, dass diese in Übereinstimmung mit der ihnen zugeschriebenen Alarmfunktion für Artgenossen (Blanchard et al., 1991; zur Übersicht siehe: Litvin et al., 2007) häufiger bei An- als bei Abwesenheit eines Artgenossen auftreten (Studie VI). Weitere soziale Faktoren, wie die Dauer seit der Trennung vom Artgenossen und die An- beziehungsweise Abwesenheit des Geruchs eines Artgenossen, wurden schließlich im Hinblick auf ihre Bedeutung für die Produktion von 50-kHz Vokalisationen geprüft (Studie III).

Zum anderen wurde untersucht, welchen Einfluss die Produktion von Rufen auf den Empfänger hat. Hierfür wurden vor allem Abspielexperimente durchgeführt. Unter Verwendung der künstlichen Präsentation von 40-kHz Vokalisationen wurde geprüft, ob Rattenmütter entgegen der Hypothese von Smotherman et al. (1974) auch ohne die Anwesenheit eines Jungtiers ein auf den Lautsprecher hin ausgerichtetes Suchverhalten zeigen (Studie I). Ferner wurde untersucht, in welchem Zusammenhang die Produktion von 60-kHz Rufen und das von den Mäusemüttern gezeigte Eintrageverhalten stehen (Studie II). Die Bedeutung von 22-kHz und 50-kHz Vokalisationen der Ratte wurde schließlich ebenfalls durch die künstliche Präsentation dieser Rufe geprüft. Es wurde in Übereinstimmung mit der Tatsache, dass 22-kHz Vokalisationen in aversiven Situationen, wie etwa nach Verabreichung eines elektrischen Schlags (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005), auftreten, wohingegen 50-kHz Vokalisationen für appetitive Situationen, wie beispielsweise Spiel (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998) und Kitzeln (Burgdorf & Panksepp, 2001; Burgdorf et al., im Druck; Mällo et al., 2007; Panksepp & Burgdorf, 1999; 2000; 2003; Schwarting et al., 2007), typisch sind, erwartet, dass diese beim Empfänger entgegengesetzte Verhaltensreaktionen auslösen. Demgemäß sollten Tiere auf 22-kHz Vokalisationen mit lokomotorischer Inhibition und Vermeidungsverhalten reagieren, wohingegen sie auf 50-kHz Vokalisationen mit lokomotorischer Aktivierung und Annäherungsverhalten reagieren sollten (Studie IV). Diese entgegengesetzten Verhaltensreaktionen sollten von einer unterschiedlich ausgeprägten Hirnaktivität begleitet

werden (Studie V). So sollten 22-kHz Vokalisationen Hirnregionen aktivieren, die an der Regulation von Angst und Furcht beteiligt sind, wie beispielsweise die Amygdala (LeDoux, 2000), wohingegen 50-kHz Vokalisationen Strukturen aktivieren sollten, die im Allgemeinen mit Belohnungsprozessen in Zusammenhang stehen, wie beispielsweise der Nucleus accumbens (Schultz et al., 1997; Wise, 1996).

VORGELEGTE ARBEITEN
- DEUTSCHE ZUSAMMENFASSUNG

Studie I

Frühkindliche Faktoren haben einen langanhaltenden Einfluss auf die Entwicklung des an der Regulation von Emotionen beteiligten neuronalen Systems. Insbesondere der soziale Faktor der maternalen Fürsorge spielt eine erhebliche Rolle (zur Übersicht siehe: Gordon & Hen, 2004; Zhang et al., 2006). So konnte am Tiermodell der Ratte gezeigt werden, dass Unterschiede im Ausmaß der in der frühsten Kindheit erfahrenen maternalen Pflege einen erheblichen Einfluss auf das angst-ähnliche Verhalten haben können. Adulte Ratten, die von ihrer Mutter viel Zuwendung erfahren hatten, zeigen ein ausgeprägteres Explorationsverhalten und weniger angst-ähnliches Verhalten als Tiere, die wenig Zuwendung erfahren hatten (Caldji et al., 1998; Francis et al. 1999; Menard et al., 2004; Menard & Hakvoort, 2007; Zhang et al., 2005). Ein ausgeprägtes Maß an maternaler Pflege hat demnach einen langanhaltenden anxiolytischen Effekt. Bemerkenswerterweise werden diese Unterschiede im Verhalten begleitet von Veränderungen der Hypothalamus-Hypophysen-Nebennieren-Achse (Liu et al., 1997) und Rezeptorsystemen, die an der zentralnervösen Regulation von Angst und Furcht beteiligt sind (Caldji et al., 1998). Da sowohl 40-kHz Vokalisationen im Jungtier (Hofer, 1996) als auch 22-kHz Vokalisationen im adulten Tier (Jelen et al., 2003) als Maße der Angst verstanden werden, sollten diese in Abhängigkeit der erfahrenen maternalen

Zuwendung unterschiedlich stark auftreten. Tiere, die viel Zuwendung erfahren hatten, sollten weniger vokalisieren, als Tiere, die wenig Zuwendung erfahren hatten. Ferner sollte sich nicht allein der soziale Faktor der maternalen Fürsorge auf das Rufverhalten auswirken, sondern das Rufverhalten auch maternales Eintrageverhalten auslösen können. Die Befundlage hierzu ist jedoch widersprüchlich. Im Gegensatz zu Allin und Banks (1972), die ein Suchverhalten in Folge der Präsentation von 40-kHz Rufen unter Abwesenheit des Geruchs eines Jungtiers beobachten konnten, war in einer Untersuchung von Smotherman et al. (1974) die Präsenz olfaktorischer Stimuli notwendig. Ziel der folgenden Studie I war es daher zu prüfen, inwiefern die erfahrene maternale Fürsorge mit dem im jungen und adulten Tier auftretenden Vokalisations- und Angstverhalten kovariieren, und ob 40-kHz Rufe tatsächlich auch ohne Anwesenheit eines Jungtiers maternale Verhaltensweisen stimulieren können.

Hierzu wurde die maternale Fürsorge, welche die jungen Ratten (Wistar) innerhalb der ersten Lebenstage erfuhren, individuell bestimmt. Ferner wurde die Reaktion der Mütter auf die Präsentation von 40-kHz Rufen und Sinustönen unter Abwesenheit von Jungtieren erfasst. Zur Auslösung der 40-kHz Rufe im Jungtier wurden diese kurz von Mutter und Geschwister getrennt. Im Erwachsenenalter wurden dieselben Tiere dann hinsichtlich lokomotorischer Aktivität in der Aktivitätsbox [*activity box*], angst-ähnlichem Verhalten im erhöhten Pluslabyrinth und während der Furchtkonditionierung untersucht. Als abhängige Variablen dienten das sichtbare Verhalten und die 40-kHz beziehungsweise 22-kHz Vokalisationen.

Die Ergebnisse zeigen, dass sich Tiere in Abhängigkeit der erfahrenen maternalen Fürsorge hinsichtlich der Emission von 40-kHz Rufen in der Kindheit und der Emission von 22-kHz Rufen im Erwachsenenalter unterschieden. Jungtiere, welche ein hohes Maß an Fürsorge erhalten hatten, emittierten weniger 40-kHz Rufe in Isolation als Jungtiere, welche ein geringes Maß an Fürsorge erfahren hatten. Bemerkenswerterweise waren auch verschiedene Rufmerkmale abhängig von der erfahrenen Pflege. So waren die Rufe stark umsorgter Tiere leiser und kürzer als die Rufe wenig umsorgter Tiere. Jedoch waren die Rufe stark umsorgter Tiere stärker frequenzmoduliert als jene von wenig umsorgten Tieren. Unterschiede im Verhalten der Jungtiere waren kaum zu beobachten, obwohl die Emission von 40-kHz Rufen stark positiv mit der lokomotorischen Aktivität der Tiere korrelierte. Im Erwachsenenalter unterschieden sich stark und wenig umsorgte Tiere ebenfalls primär in ihrem Vokalisationsverhalten. Tiere, welche innerhalb der ersten Lebenstage ein hohes Maß an Fürsorge erhalten hatten, emittierten mehr 22-kHz Rufe während der Furchtkonditionierung als wenig umsorgte Tiere. Die Emission von 22-kHz Vokalisationen korrelierte positiv mit der Zeit, welche die Tiere in Verhaltensstarre verbrachten. Hinsichtlich

der kommunikativen Bedeutung der 40-kHz Rufe konnte gezeigt werden, dass Mütter in Reaktion auf deren Darbietung auch in Abwesenheit von Jungtieren mit einem auf den Lautsprecher hin ausgerichteten Suchverhalten reagieren. 40-kHz Sinustöne hingegen erwiesen sich als nicht effektiv.

Die Ergebnisse stützen die Annahme, dass die erfahrene maternale Fürsorge einen Einfluss auf das Vokalisationsverhalten hat. Die am Jungtier gewonnenen Ergebnisse, dass bei einer ausgeprägten maternalen Pflege 40-kHz Rufe vergleichsweise selten auftreten, stehen in Einklang mit den an adulten Tieren erhobenen Befunden, die zeigen konnten, dass eine ausgeprägte maternale Fürsorge anxiolytisch wirkt (Caldji et al., 1998; Francis et al. 1999; Menard et al., 2004; Menard & Hakvoort, 2007; Zhang et al., 2005). Im Widerspruch mit diesen Befunden steht aber die Beobachtung, dass im Erwachsenenalter Tiere, die von ihren Müttern innerhalb der ersten Lebenstage stark umsorgt wurden, mehr 22-kHz Rufe aussandten als wenig umsorgte Tiere. Möglicherweise beeinflusst die maternale Fürsorge nicht die Auftretenswahrscheinlichkeit angst-ähnlichen Verhaltens per se, sondern das Verhältnis zwischen aktiven und passiven Stressbewältigungsstrategien. Tatsächlich geht die Emission von 40-kHz Rufen mit einer ausgeprägten lokomotorischen Aktivität einher (Hofer & Shair, 1978) wobei die Emission von 22-kHz Rufen stark mit der in aversiven Situationen gezeigten Verhaltensstarre kovariiert (Burgdorf et al., im Druck; Choi & Brown, 2003; Wöhr et al., 2005). Es erscheint also möglich, dass eine ausgeprägte maternale Pflege eher einen passiven Stressbewältigungsstil begünstigt als einen aktiven. Schließlich belegen die Ergebnisse, dass 40-kHz Rufe auch unter Abwesenheit eines Jungtieres auf die Schallquelle hin ausgerichtetes maternales Suchverhalten induzieren können. Damit kontrastieren die vorliegenden Ergebnisse mit der von Smotherman et al. (1974) gemachten Beobachtung, dass Mütter nur dann gerichtetes Suchverhalten zeigen, wenn neben den 40-kHz Rufen auch der Geruch eines Jungtieres präsentiert wird. Sie stimmen jedoch mit der von Allin und Banks (1972) gemachten Beobachtung überein, dass diese Rufe der Lokalisation von aus dem Nest entfernten Jungtieren dienen können.

Studie II

In Studie I konnte gezeigt werden, dass die durch die Mutter in den ersten Lebenstagen erfahrene maternale Fürsorge negativ mit der Emission von 40-kHz Vokalisationen kovariierte. Aufgrund des korrelativen Ansatzes konnte jedoch nicht geklärt werden, inwiefern diese Korrelation durch den genetischen Hintergrund des Jungtiers oder durch den Umweltfaktor maternale Fürsorge verursacht wurde. In Zuchtstudien konnte gezeigt werden, dass genetische Faktoren für die Emission von 40-kHz Rufen von großer Bedeutung sind (Brunelli & Hofer, 2007; Brunelli, 2005; Brunelli et al., 1997; 2001; 2002; Hofer et al., 2001; Shair et al., 2000; Zimmerberg et al., 2005). Auch die Emission von 60-kHz Rufen bei der Maus ist stark von genetischen Faktoren abhängig – wie genetische Analysen (Hahn et al., 1987; 1997; 1998; Hahn & Schanz, 2002; Roubertoux et al., 1996; Thornton et al., 2005) und Studien an Knock-Out und Knock-In Tieren zeigen (Brunner et al., 1999; El-Khodor et al., 2004; Fujita et al., 2008; Groszer et al., 2008; Moles et al., 2004; Scattoni et al., 2007; Shu et al., 2005; Weller et al., 2003; Winslow et al., 2000). Bei Maus und Ratte scheinen jedoch auch Umweltfaktoren, insbesondere die maternale Pflege, einen Einfluss auf die Emission isolations-induzierter Vokalisationen zu haben (D'Amato & Populin, 1987; D'Amato et al., 2005; Darnaudery et al., 2004). Ziel der folgenden Studie II

war es daher, die Bedeutung genetischer Faktoren von der Bedeutung der Umweltfaktoren hinsichtlich der Emission isolations-induzierter Vokalisationen abzugrenzen.

Hierzu wurden die isolations-induzierten Ultraschallvokalisationen zweier Mäusestämme (C57BL/6JOlaHsd [B6JOla] und C57BL/6NCrl [B6N]) verglichen. Zur Aufklärung der Bedeutung genetischer und epigenetischer Faktoren wurde ein Emryotransfer durchgeführt. Blastozysten des jeweiligen Stammes wurden entweder in ein Weibchen desselben oder des anderen Stammes verpflanzt. Somit unterschieden sich die Jungtiere eines Stammes in Abhängigkeit der Mutter hinsichtlich der prä-, peri- und postnatalen Umwelt. Um isolations-induzierte Ultraschallvokalisationen zu induzieren, wurden die Tiere einige Tage nach der Geburt kurz von Mutter und Geschwistern getrennt. Ferner wurde das Fürsorgeverhalten der Mütter bestimmt. Als abhängige Variablen dienten die isolations-induzierten Ultraschallvokalisationen und die Zeit, die die Mütter benötigten bis sie begannen, aus dem Nest entnommene Jungtiere in das Nest einzutragen.

Die Ergebnisse zeigen, dass sich B6Jola und B6N Mäuse hinsichtlich der Anzahl und der Art isolations-induzierter Ultraschallvokalisationen unterschieden. Diese Unterschiede basieren nur teilweise auf dem genetischen Hintergrund der Tiere. Mittels Embryotransfer konnte gezeigt werden, dass die Anzahl der emittierten Rufe stark von der Mutter beziehungsweise von der Interaktion zwischen Mutter und Jungtier abhängig ist. Ferner wirkte sich der Faktor Mutter auch in bedeutsamer Weise auf die Lautstärke der Rufe aus. Ruffrequenz und Frequenzmodulation waren hingegen abhängig vom genetischen Hintergrund des Jungtieres und unabhängig von der Mutter. In Übereinstimmung mit der Interaktion zwischen Genotyp und Mutter im Hinblick auf die Rufanzahl konnte gezeigt werden, dass sich das Eintrageverhalten der Mütter invers zum Vokalisationsverhalten der Jungtiere verhält. B6N Mütter trugen B6N Jungtiere schneller ein als B6Jola Jungtiere, wobei die von B6N Müttern aufgezogenen B6N Jungtiere auch mehr vokalisierten als die B6JOla Jungtiere. Umgekehrt verhielt es sich bei B6JOla Müttern.

Die Ergebnisse stützen daher die Annahme, dass die erfahrene maternale Fürsorge einen Einfluss auf das Vokalisationsverhalten hat. So zeigen die Ergebnisse deutlich, dass Rufanzahl und Lautstärke der Rufe stark von epigenetischen Faktoren beeinflusst werden. Dies steht in Einklang mit Befunden einer anderen Emryotransferstudie, wo die große Bedeutung epigenetischer Faktoren für das adulte angst-ähnliche Verhalten nachgewiesen werden konnte (Francis et al., 2003). Zukünftige Studien werden zu klären haben, welches Gewicht prä-, peri- und postnatale Faktoren jeweils unabhängig voneinander haben. In Einklang mit der Vermutung, dass für die beobachteten epigenetischen Effekte Unterschiede

in der maternalen Pflege ursächlich sind, steht die bemerkenswerte Übereinstimmung hinsichtlich der betroffenen Rufparameter bei Maus und Ratte (Studie I). Trotz unterschiedlicher experimenteller Vorgehensweisen erwiesen sich Rufanzahl und Lautstärke der Rufe in beiden Fällen als sensitiv für Veränderungen in der frühkindlichen Umwelt. Gleichwohl verweist die Interaktion zwischen genetischem Hintergrund und Mutter auf die Bedeutung des Genotyps. Andere Rufparameter, wie Frequenz und Frequenzmodulation, waren sogar allein vom Genotyp abhängig. Eine starke Bedeutung des Genotyps für die Emission von Ultraschallvokalisationen bei der Maus konnte wiederholt nachgewiesen werden (Hahn et al., 1987; 1997; 1998; Hahn & Schanz, 2002; Roubertoux et al., 1996; Thornton et al., 2005). Aufgrund der hohen genetischen Übereinstimmung zwischen B6Jola und B6N Mäusen, ist das Vorliegen einer Spontanmutation auf Chromosom 6 bei B6JOla Mäusen besonders bemerkenswert. Diese Spontanmutation führte unter anderem zu einem Fehlen des Proteins alpha-Synuclein (Chen et al., 2002; Siegmund et al., 2005; Specht & Schoepfer, 2001; 2004) und es erscheint daher naheliegend, dass das Fehlen von alpha-synuclein für die Unterschiede in der Rufproduktion verantwortlich ist.

Studie III

In einer Vielzahl von Untersuchungen konnte gezeigt werden, dass 50-kHz Vokalisationen nicht allein in appetitiven Situationen, wie Spiel (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998) oder Paarung (Barfield et al., 1979; Bialy et al., 2000; Burgdorf et al., im Druck; Geyer & Barfield, 1978; McGinnis & Vakulenko, 2003; McIntosh et al., 1978; Sales, 1972b; White & Barfield, 1990; White et al., 1990) auftreten können, sondern auch in wahrscheinlich non-appetitiven oder gar aversiven Situationen. So senden Kontrolltiere in einer Vielzahl von Bedingungen 50-kHz Rufe aus (Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Knutson et al., 1999; Thompson et al., 2006; Wintink & Brudzynski, 2001). Auch sind 50-kHz Rufe in aggressiven Auseinandersetzungen (Burgdorf et al., im Druck; Haney & Miczek, 1994; Sales, 1972a; Thomas et al., 1983; Tornatzky & Miczek, 1994; 1995; Vivian & Miczek, 1993a; 1993b) und während Drogenentzug (Vivian & Miczek, 1991) zu beobachten. Bemerkenswerterweise kann die Auftretenshäufigkeit dieser Vokalisationen durch anxiolytische Substanzen, wie etwa Diazepam, abgesenkt werden (Tornatzky & Miczek, 1995). In einer kürzlich durchgeführten Untersuchung konnte ferner gezeigt werden, dass bereits das Versetzen eines Tieres in einen neuen Käfig zu 50-kHz Vokalisationen führt (Schwarting et al., 2007). Ziel der folgenden

Studie III war es daher, herauszufinden, von welchen Faktoren die Emission von 50-kHz Rufen in non-appetitiven Situationen, wie etwa Käfigexposition nach Versetzen, abhängt.

Hierzu wurden im ersten Experiment zwei Gruppen von Ratten (Wistar) untersucht. Eine der beiden Gruppen wurde über mehrere Wochen täglich in einer appetitiven Diskriminationsaufgabe trainiert, wohingegen die andere Gruppe nicht trainiert wurde. Beide Gruppen wurden innerhalb der letzten Trainingswoche an drei aneinanderfolgenden Tagen hinsichtlich ihrer 50-kHz Vokalisationen nach Trennung vom Artgenossen durch Versetzen in einen nach einem bekannten Artgenossen riechenden Käfig untersucht. Wobei die Tiere, die nicht in der Diskriminationsaufgabe trainiert wurde, sofort nach Trennung vom Artgenossen getestet wurden, wohingegen jene, welche trainiert wurden, erst nach dem Training getestet wurden. Am zweiten Tag wurde jeweils die Hälfte der Tiere in einem neuen Käfig ohne Geruch des Artgenossen getestet. Abschließend wurden sie hinsichtlich ihres angst-ähnlichen Verhaltens im Offenfeld und erhöhten Pluslabyrinth untersucht. Als abhängige Variablen dienten die 50-kHz Vokalisationen und das in den folgenden Verhaltentests gezeigte angst-ähnliche Verhalten. Im zweiten Experiment wurde geprüft, ob nur das Tier, welches in einen neuen Käfig versetzt wurde, vokalisiert, oder auch jenes, welches im Heimkäfig allein zurückblieb. Als abhängige Variable dienten die 50-kHz Vokalisationen beider Gruppen.

Die Ergebnisse des ersten Experiments zeigen, dass Tiere, die nicht in der appetitiven Diskriminationsaufgabe trainiert wurden, mehr vokalisierten, als jene, die trainiert wurden. Die nicht trainierten Tiere emittierten vor allem in den ersten Minuten nach Versetzen meist flache 50-kHz Rufe. Die An- beziehungsweise Abwesenheit von Geruch des Artgenossen hatte nur einen geringen Einfluss auf das Vokalisationsverhalten. Zusammenhänge zwischen 50-kHz Vokalisationen und dem angst-ähnlichen Verhalten im Offenfeld und erhöhten Pluslabyrinth waren kaum gegeben. Im zweiten Experiment konnte gezeigt werden, dass tatsächlich beide Tiere vokalisierten, also sowohl jenes, das von seinem Artgenossen getrennt und in einen neuen Käfig versetzt wurde, als auch jenes, welches nach der Trennung im Heimkäfig verblieb. Interessanterweise vokalisierte das Tier, welches im Heimkäfig zurückblieb, mehr als jenes, das in einen neuen Käfig versetzt wurde.

Die vorliegenden Ergebnisse zeigen, dass 50-kHz Vokalisationen in Situationen auftreten können, die wahrscheinlich keinen appetitiven Charakter aufweisen. Damit stehen die Befunde in Einklang mit der Beobachtung von 50-kHz Rufen bei Kontrolltieren (Brudzynski & Pniak, 2002; Knutson et al., 1999; McGinnis & Vakulenko, 2003; Thompson et al., 2006; Wintink & Brudzynski, 2001) und Kämpfen (Burgdorf et al., im Druck; Haney & Miczek, 1994; Sales, 1972a; Thomas et al., 1983; Tornatzky & Miczek, 1994; 1995; Vivian &

Miczek, 1993a; 1993b). In Übereinstimmung mit einer Untersuchung von Schwarting et al. (2007) konnten 50-kHz Vokalisationen durch das Versetzen des Tieres in eine neue Umgebung ausgelöst werden. Gegen die Annahme, dass dieses Versetzen in eine neue Umgebung zentraler Auslöser des Vokalisationsverhaltens ist, spricht jedoch die Tatsache, dass kaum 50-kHz Rufe bei Tieren beobachtet werden konnten, welche zuvor in einer appetitiven Diskriminationsaufgabe trainiert wurden, wohingegen Tiere, die nicht trainiert wurden, ein ausgeprägtes Vokalisationsverhalten zeigen. Gemäß der Hypothese, dass diese Rufe allein in Antizipation von Verstärkern auftreten, hätte man ein umgekehrtes Ergebnis erwarten müssen. Auffälliger Unterschied zwischen beiden Gruppen ist die seit der Trennung vom Artgenossen vergangene Zeit während der Messung von 50-kHz Vokalisationen. Tiere, die nicht in der Diskriminationsaufgabe trainiert wurden, wurden sofort nach Trennung vom Artgenossen getestet, nicht aber jene, welche trainiert wurden. Dieser Unterschied verweist auf eine mögliche Bedeutung der Trennung vom Artgenossen, genauer auf eine mögliche Bedeutung der seit der Trennung vergangenen Zeit, für die Emission von 50-kHz Rufen. Diese Beobachtung kann am ehesten damit erklärt werden, dass 50-kHz Rufe direkt nach der Trennung ausgesandt werden, um den Kontakt mit dem Artgenossen wieder herzustellen. Tatsächlich vokalisierten die Tiere, welche nicht trainiert wurden, vor allem zu Beginn der Messung. Sollte die Annahme, dass die Trennung vom Artgenossen den Auslöser des Vokalisationsverhaltens darstellt, richtig sein, so würde man erwarten, dass nicht allein die Tiere, welche nach der Trennung in einen neuen Käfig versetzt wurden, vokalisieren, sondern auch jene Tiere, welche allein im Heimkäfig zurückbleiben. Dies war auch tatsächlich der Fall. Der Umstand, dass die im Heimkäfig verbliebenen Tiere sogar mehr vokalisierten, als jene, welche versetzt wurden, kann auf die Tatsache zurückgeführt werden, dass neue Situationen anxiogen wirken können. Bekanntermaßen wirken anxiogene Stimuli, wie beispielsweise helles Licht (Knutson et al., 1998; Panksepp & Burgdorf, 1999), Geruch einer Katze (Panksepp & Burgdorf, 1999) oder ein Hinweisreiz auf einen elektrischen Schlag (Burgdorf et al., 2000), hemmend auf die Emission von 50-kHz Rufen.

Studie IV

Die Tatsache, dass Ratten 50-kHz Vokalisationen in Reaktion auf die Trennung von einem Artgenossen aussenden (Studie III), legt die Vermutung nahe, dass diese Vokalisationen der Aufrechterhaltung von Sozialkontakt dienen. Tatsächlich konnte gezeigt werden, dass Tiere mehr Zeit mit anderen Tieren verbringen, die viel vokalisieren, als mit Tieren, welche wenig vokalisieren (Panksepp et al., 2002). Außerdem zeigen Tiere, bei welchen experimentell das Hörvermögen ausgeschaltet wurde, Auffälligkeiten im Bereich des Sozialverhaltens (Siviy & Panksepp, 1987). Allerdings zeigten Tiere in Reaktion auf die künstliche Darbietung von 50-kHz Rufen kein Annäherungsverhalten (Burman et al., 2007; Endres et al., 2007). Auch hinsichtlich der kommunikativen Bedeutung von 22-kHz Vokalisationen liegt keine einheitliche Befundlage vor. Studien, in denen Tiere deutliche Verhaltensreaktionen in Folge der Darbietung von 20-kHz Sinustönen zeigen (Beckett et al., 1996; 1997; Commissaris et al., 1998; 2000; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Voits et al., 1999), kontrastieren mit Untersuchungen, in denen unter Verwendung natürlicher Signale keine (Bang et al., im Druck; Lindquist et al., 2004; Tankhiwale et al., 2007) oder lediglich schwache Verhaltensreaktionen (Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991) beobachtet wurden. Ziel der

folgenden Studie IV war es daher, die kommunikative Bedeutsamkeit von 22-kHz und 50-kHz Rufen durch die Erfassung der Verhaltensreaktion des Empfängers zu prüfen.

Hierzu wurden drei Experimente mit Ratten (Wistar) durchgeführt. Im ersten Experiment wurde juvenilen Tieren 22-kHz und 50-kHz Rufe präsentiert. Im zweiten Experiment wurde juvenilen Tieren natürliche 50-kHz Rufe sowie artifizielle 50-kHz Rufe ohne Frequenzmodulation präsentiert. Im dritten Experiment wurde dieses Design übernommen und beide Stimuli adulten Tieren präsentiert. Als Kontrollsignal diente weißes Rauschen. Alle Tiere wurden jeweils allen akustischen Stimuli in randomisierter Reihenfolge ausgesetzt. Als abhängige Variablen wurden das Vokalisationsverhalten der Tiere und deren sichtbares Verhalten erhoben. Um den Anreizcharakter der Stimuli zu prüfen, wurde die Anzahl der Eintritte in den Bereich vor dem Lautsprecher und die dort während der Stimuluspräsentation verbrachte Zeit erhoben. Ferner wurde die zurückgelegte Wegstrecke als Maß für die lokomotorische Aktivität erhoben.

Die Ergebnisse zeigen, dass 50-kHz Vokalisationen motorische Aktivität induzierten, welche auf die Schallquelle gerichtet ist. Dies war in besonders ausgeprägtem Maße für die juvenilen Tiere zutreffend. Bei adulten Tieren war die Annäherung an die Schallquelle geringer ausgeprägt. Das Annäherungsverhalten konnte bei juvenilen und adulten Tieren nicht nur durch natürliche 50-kHz Vokalisationen ausgelöst werden, sondern auch durch artifiziell verfremdete 50-kHz Rufe ohne Frequenzmodulation. 22-kHz Vokalisationen dagegen inhibierten tendenziell lokomotorische Aktivität. Vermeidungsverhalten konnte jedoch nicht beobachtet werden. Ultraschallvokalisationen in Reaktion auf die Präsentation von Vokalisationen traten bei juvenilen und adulten Tieren nur gelegentlich auf.

Die vorliegenden Ergebnisse stützen die aus Studie III abgeleitete Annahme, dass 50-kHz Vokalisationen der Aufrechterhaltung von Sozialkontakt dienen. Erstmals konnte gezeigt werden, dass juvenile und adulte Tiere in Reaktion auf die Präsentation von 50-kHz Rufen Annäherungsverhalten zeigen. Die deutlichen Effekte, welche in der vorliegenden Arbeit vorgefunden wurden, kontrastieren mit den fehlenden Effekten in anderen Untersuchungen (Burman et al., 2007; Endres et al., 2007). Mögliche Ursache hierfür könnte unter anderem die genutzte Technik zum Präsentieren der akustischen Stimuli sein. Auch die Position des Lautsprechers könnte hierfür relevant sein, da kein Annäherungsverhalten beobachtet werden kann, wenn der Lautsprecher wie in der Studie von Endres et al. (2007) über den Tieren angebracht ist. Die vorliegende Studie wird jedoch durch eine Untersuchung gestützt, in welcher Ratten die Möglichkeit hatten, sich 50-kHz Vokalisationen mittels Durchbrechen einer Lichtschranke selbst zu verabreichen (Burgdorf et al., im Druck). Ratten durchbrachen

die Lichtschranke, welche mit der Präsentation von 50-kHz Rufen gekoppelt war, häufiger als eine Lichtschranke, die nicht mit der Präsentation von Vokalisationen gekoppelt war. Bemerkenswerterweise konnte in der vorliegenden Arbeit ferner beobachtet werden, dass juvenile Tiere stärker auf die Präsentation von 50-kHz Rufen reagieren als adulte Tiere. Dies stimmt mit der Tatsache überein, dass juvenile Tiere selbst eher 50-kHz Rufe aussenden (Panksepp & Burgdorf, 1999). Die Annahme jedoch, dass die Frequenzmodulation ein zentrales Merkmal der 50-kHz Rufe darstellt (Burgdorf & Panksepp, 2006; Burgdorf et al., 2007; im Druck), konnte im Hinblick auf deren kommunikative Bedeutung nicht bestätigt werden. Sowohl juvenile als auch adulte Tiere zeigten Annäherungsverhalten bei Präsentation von artifiziell verfremdete 50-kHz Rufe ohne Frequenzmodulation. Im Vergleich zu der durch 50-kHz Vokalisationen induzierten Verhaltensänderung waren die Effekte der Präsentation von 22-kHz Rufen relativ schwach. Dies steht in Einklang mit der Beobachtung schwacher Verhaltenseffekte in adulten Tieren unter Verwendung natürlicher 22-kHz Rufe (Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991). Es muss daher angenommen werden, dass die beobachteten starken Effekte, welche durch die Präsentation von künstlichen 20-kHz Sinustönen erzielt werden können (Beckett et al., 1996; 1997; Commissaris et al., 1998; 2000; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Voits et al., 1999), auf andere Merkmale als die Frequenz und der hierin potentiell kodierten Bedeutsamkeit des präsentierten Stimulus zurückzuführen sind.

Studie V

22-kHz und 50-kHz Vokalisationen lösen beim Empfänger entgegengesetzte Verhaltensreaktionen aus (Studie IV). In Übereinstimmung mit den Situationen, in welchen die Rufe auftreten, hemmen 22-kHz Rufe lokomotorische Aktivität, wohingegen 50-kHz Rufe lokomotorische Aktivität induzieren, welche auf die Schallquelle gerichtet ist. Über die Hirnregionen, welche an der Verarbeitung dieser Vokalisationen und der Steuerung der darauf folgenden Verhaltensreaktion beteiligt sind, ist bisher wenig bekannt. In Studien unter Verwendung künstlicher 20-kHz Sinustöne konnte man mit Hilfe immunohistochemischer Analyse nachweisen, dass diese Amygdala und zentrales Höhlengrau aktivieren (Beckett et al., 1997; Neophytou et al., 2000), die von zentraler Bedeutung für die Regulation von Angst und Furcht sind (LeDoux, 2000). Kritisch ist allerdings anzumerken, dass in diesen Studien kein akustisches Kontrollsignal verwandt wurde. Läsionsstudien konnten ferner zeigen, dass der perirhinale Cortex an der Verarbeitung von 22-kHz Rufen beteiligt ist. Nach Ausschalten dieser Struktur lernten Tiere nicht länger, diese Vokalisationen (CS) mit einem elektrischen Schlag (US) zu assoziieren (Lindquist et al., 2004). Einzelzellableitungen in dieser Region bestätigten die Beteiligung dieser Hirnregion an der Verarbeitung von 22-kHz Rufen (Allen et al., 2007; Furtak et al., 2007). Über die Hirnregionen, die an der Verarbeitung von 50-kHz

Rufen beteiligt sind, ist hingegen noch nichts bekannt. Man kann hierbei lediglich Vermutungen aufstellen. So ist bekannt, dass Spielverhalten, welches mit der Produktion von 50-kHz Vokalisationen einhergeht (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998), zu einer Aktivität im inferioren Colliculus, Tectum, zentralen Höhlengrau, parietalen Cortex, sowie dem dorsalen und ventralen Striatum führt (Gordon et al., 2002). Allerdings ist es nicht möglich zwischen der durch die taktile Stimulation des Spiels und jener durch die akustische Stimulation induzierten Aktivität zu unterscheiden. Der in Studie IV nachgewiesene hohe positive Anreizcharakter lässt ferner vermuten, dass es sich bei den durch 50-kHz Rufe aktivierten Regionen, um jene handelt, die mit Belohnungsprozessen in Zusammenhang gebracht werden, wie etwa der Nucleus accumbens (Schultz et al., 1997; Wise, 1996). Ziel der folgenden Studie V war es daher, die durch die Präsentation von 22-kHz und 50-kHz Rufen aktivierten Hirnregionen auszumachen und deren Aktivität zu beschreiben.

Hierzu wurden Ratten (Wistar) entweder 22-kHz oder 50-kHz Vokalisationen ausgesetzt. Eine weitere Gruppe von Tieren wurde dem Kontrollstimulus weißes Rauschen ausgesetzt, um zwischen jener Aktivierung, die lediglich durch akustische Stimulation bedingt ist, und jener Aktivierung, die in der kommunikativen Bedeutung der Signale begründet ist, differenzieren zu können. Schließlich wurde eine weitere Kontrollgruppe genutzt, bei welcher keine akustische Präsentation erfolgte, um die Aktivität, die lediglich durch die Testumgebung induziert wurde, beschreiben zu können. Nach Abschluss der Stimuluspräsentation wurden die Tiere getötet und die Gehirne entnommen. Als abhängiges Maß der Hirnaktivität wurde schließlich in ausgewählten Regionen die Anzahl c-fos positiver Zellen bestimmt. Das transient exprimierte Gen c-fos gehört zur Familie der sogenannten unmittelbar auftretenden frühen Gene [*immediate early genes*], welche innerhalb weniger Minuten nach Stimulation der Zelle transkribiert werden. Die Stimulation erfolgt durch eine Vielzahl extrazellulärer Prozesse, wie etwa durch an der Zelle eintreffende Aktionspotentiale. Die Transkription von c-fos ist daher stark mit der Aktivität der Zelle korreliert. Die unmittelbar auftretenden frühen Gene fungieren selbst größtenteils als Transkriptionsfaktoren, welche regulativ auf die Expression sogenannter verzögert auftretender Reaktionsgene [*delayed response genes*] einwirken, was letztlich zu einer Modulation langanhaltender cytologischer Veränderungen, wie Synaptogenese, führt. Das Gen c-fos ist unter Verwendung spezieller immunohistochemischer Methoden bereits wenige Minuten nach Aktivität der Zelle auf Peptidebene nachweisbar. Es ist daher möglich mit zellspezifischen Auflösungsvermögen Auskunft darüber zu geben, welche Zelle in einem bestimmten Zeitfenster, etwa während einer experimentellen Manipulation, aktiv war (Morgan & Corran, 1991).

Die Ergebnisse zeigen, dass 22-kHz und 50-kHz Vokalisationen erwartungsgemäß den auditorischen Cortex aktivierten. Dessen Aktivität war am stärksten bei der Präsentation von 22-kHz Rufen. 50-kHz Rufe induzierten eine vergleichsweise schwache Aktivität. Darüber hinaus führten beide Signale jedoch zu einem stark unterschiedlichen Erregungsmuster. 22-kHz Rufe induzierten Aktivität im perirhinalen Cortex, ectorhinalen Cortex, in der lateralen und basolateralen Amygdala sowie im rostralen Bereich des zentralen Höhlengraus. Eine Absenkung der Aktivität in Reaktion auf 22-kHz Vokalisationen konnte im paraventriculären Nucleus des Thalamus und den dorsalen Raphé-Kernen nachgewiesen werden. 50-kHz Rufe hingegen führten zu einer erhöhten Aktivität im frontalen Cortex und Nucleus accumbens. Eine Aktivitätsabnahme erfolgte in Reaktion auf 50-kHz Vokalisationen im zentralen Kern der Amygdala, dem lateralen Nucleus habenularis und den dorsalen Raphé-Kernen.

Die vorliegenden Ergebnisse zeigen, dass 22-kHz und 50-kHz Vokalisationen eine unterschiedliche Hirnaktivität mit teilweise entgegengesetzten Effekten, wie im Falle der Amygdala, zu induzieren vermögen. 22-kHz Rufe aktivieren den perirhinalen Cortex, den ectorihnalen Cortex, die Amygdala und das zentrale Höhlengrau. Die Übereinstimmung der durch natürliche 22-kHz Rufe induzierten Aktivität und jener, welche durch das Präsentieren von 20-kHz Sinustönen erzeugt wurde, ist hoch (Beckett et al., 1997; Neophytou et al., 2000). So wurde in beiden Studien eine erhöhte Aktivität in der Amygdala und zentralem Höhlengrau beobachtet. Augenscheinlich aktivieren 22-kHz Vokalisationen Hirnregionen, die im Zusammenhang mit der Regulation von Angst und Furcht stehen (LeDoux, 2000). Dies stimmt mit der durch sie induzierten Verhaltensreaktion überein (Studie IV; Beckett et al., 1996; 1997; Brudzynski & Chiu, 1995; Burman et al., 2007; Commissaris et al., 1998; 2000; Endres et al., 2007; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Sales, 1991; Voits et al., 1999). 50-kHz Rufe aktivieren in Übereinstimmung mit der beobachtbaren Verhaltensreaktion (Studie IV) Hirnregionen, wie den Nucleus accumbens, welche mit Belohnungsprozessen assoziiert sind (Schultz et al., 1997; Wise, 1996). Bekanntlich führt die direkte Verabreichung des Dopamin-Agonisten Amphetamin in den Nucleus accumbens zu 50-kHz Vokalisationen (Burgdorf et al., 2001; 2007; Knutson et al., 1999; Thompson et al., 2006), ebenso wie die elektrische Stimulation dopaminerger, den Nucleus accumbens innervierender Bahnen (Burgdorf et al., 2000; 2007). Eine Läsion des ventralen Tegmentums führt hingegen zu einer Hemmung von 50-kHz Rufen, wie auch die Gabe des Dopmain-Antagonisten Flupenthixol (Burgdorf et al., 2007). Ferner geht Spielverhalten mit einer erhöhten Aktivität im Nucleus accumbens einher (Gordon et al., 2002). Beim Spielen treten im Allgemeinen 50-kHz Vokalisationen auf (Brunelli et al., 2006; Knutson et al., 1998).

Studie VI

Es wurde angenommen, dass 22-kHz Vokalisationen dazu dienen Artgenossen zu warnen (Blanchard et al., 1991). Tatsächlich konnte in einigen Studien in Reaktion auf Präsentationen von natürlichen 22-kHz Vokalisationen eine Änderung des Verhaltens beim Empfänger beobachtet werden (Studie IV; Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991). Diese Verhaltensänderung wird begleitet von Hirnaktivität in Regionen, die im Zusammenhang mit der Regulation von Angst und Furcht stehen (Studie V). In Übereinstimmung mit einer Alarmfunktion für Artgenossen sollte man ferner erwarten, dass die Produktion von Rufen abhängig ist von der Anwesenheit von Artgenossen. In Studien an einer Vielzahl von Tierarten konnte gezeigt werden, dass Alarmrufe tatsächlich häufig nur dann auftreten, wenn ein Artgenosse anwesend ist (Blumstein et al., 1997; Cheney & Seyfarth, 1985; Evans & Marler, 1991; Gyger et al., 1986; Hoogland, 1983; 1996; Karakashian et al., 1988; Ridley et al., 2007; Roux et al., 2008; Sullivan, 1985; Wich & Sterck, 2003). In der Ratte liegt bisher jedoch lediglich eine Studie vor, welche einen solchen Zusammenhang nahe legt (Blanchard et al., 1991). Da in dieser Studie jedoch nicht allein die Anwesenheit eines Artgenossen variiert wurde, sondern auch zugleich Testumgebung und Haltungsbedingung, erscheint es möglich, dass diese kovariierten Faktoren ursächlich sind für

die gehäufte Beobachtung von 22-kHz Rufen in Anwesenheit von Artgenossen. Ziel der folgenden Studie VI war es daher, unter streng kontrollierten Bedingungen zu prüfen, inwiefern das Vokalisationsverhalten in aversiven Situationen tatsächlich von der Anwesenheit eines Artgenossen abhängig ist.

Hierzu wurden die Tiere einer Furchtkonditionierung unterzogen, bei der ein elektrischer Schlag (US) mehrfach mit einem Ton (CS) gepaart dargeboten wurde (Studie I; Borta et al., 2006; Schwarting et al., 2007; Wöhr et al., 2005). Die Konditionierung wurde unter 1) Abwesenheit eines Artgenossen, unter 2) Anwesenheit eines narkotisierten Artgenossen oder unter 3) Anwesenheit eines aktiven Artgenossen durchgeführt. Während der Furchtkonditionierung wurde allein das Experimentaltier und nicht das Begleittier dem US ausgesetzt. Die Begleittiere waren an allen Testtagen, also an Habituation, Konditionierung und Testung, anwesend. Als abhängige Variablen dienten neben der Emission von 22-kHz Rufen, sichtbare Verhaltensweisen, nämlich Verhaltensstarre, Aufrichten und Putzen.

Die Ergebnisse zeigen, dass Experimentaltiere aller drei Versuchsbedingungen während der Konditionierung in Reaktion auf den US begannen 22-kHz Vokalisationen auszusenden. Dies wurde begleitet von einer Abnahme aktiver Verhaltensweisen, nämlich Aufrichten und Putzen, und einer Zunahme der Verhaltensstarre. Ferner zeigten Tiere aller drei Bedingungen am Testtag 22-kHz Vokalisationen und Verhaltensstarre. Die Furchtkonditionierung erwies sich also in allen Bedingungen als effektiv. Unterschiede im sichtbaren Verhalten während Konditionierung und Testung konnten in Abhängigkeit der Bedingung nicht beobachtet werden. Die Experimentalttiere unterschieden sich auch nicht in der Anzahl der emittierten 22-kHz Vokalisationen. Die Anwesenheit eines Artgenossen beeinflusste also weder das sichtbare Verhalten noch die Emission von 22-kHz Rufen. Bemerkenswerterweise konnte jedoch gezeigt werden, dass die Emission von 22-kHz Rufen durch die Experimentaltiere während der Konditionierung positiv mit der von den Begleittieren gezeigten Verhaltensstarre korrelierte.

Die vorliegenden Ergebnisse stützen also nicht die Annahme, dass Ratten in aversiven Situationen vermehrt 22-kHz Vokalisationen in Anwesenheit von Artgenossen aussenden, denn die Emission von 22-kHz Vokalisationen war in allen drei Bedingungen ähnlich stark ausgeprägt. Die Tatsache jedoch, dass die Anzahl der 22-kHz Vokalisationen, welche das Experimentaltier produzierte, positiv mit der von den Begleittieren gezeigten Verhaltensstarre korrelierte, spricht für eine Alarmfunktion. Da es sich hierbei aber lediglich um einen korrelativen Befund handelt, ist es theoretisch möglich, dass andere Faktoren diesen Zusammenhang bewirkt haben. Gegen diese Annahme sprechen jedoch Befunde aus Studien,

in welchen natürliche 22-kHz Vokalisationen präsentiert wurden und Reaktionen auf der Ebene des Verhaltens (Studie IV; Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991) und der Hirnaktivität (Studie V) beobachtet werden konnten, die für eine Furchtinduktion im Empfänger sprechen. Zusammenfassend kann daher geurteilt werden, dass die vorliegenden Ergebnisse nicht gegen eine Alarmfunktion von 22-kHz Vokalisationen sprechen, sondern lediglich gegen die Annahme, dass deren Produktion unter aktiver Kontrolle des Tieres stehen und diese in Abhängigkeit der Anwesenheit von Artgenossen emittiert werden.

VERÖFFENTLICHUNGEN

Studie I

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Maternal Care, Isolation-Induced Infant Ultrasonic Calling, and Their Relations to Adult Anxiety-Related Behavior in the Rat

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In the rat, variations in maternal care affect the development of stable individual differences in anxiety-related behavior. Here, it was asked whether such experience-dependent differences can be detected already during early life. As a measure for anxiety in pups, isolation-induced ultrasonic vocalizations were used, and their dependency on different maternal behaviors, namely licking, retrieval behavior, and responsiveness to playback of pup calls, was tested. Consistent with reported differences of adult rats with high or low levels of maternal care experienced, the rarely licked offspring appeared to be more anxious, since they emitted more calls when separated from their mother and litter. Based on these findings, it was examined whether infant calling can be used as a predictor of adult anxiety-related behavior. Results show that infant call emission was negatively correlated with immobility and calling during fear conditioning. These relationships seem to be mediated at least partly by maternal care. In total, measuring ultrasonic vocalizations can provide information about an affective trait of infant and adult rats, which gives the opportunity to study the development of emotionality from early life onward.

Keywords: ultrasonic vocalization (USV), playback of 40-kHz calls, maternal licking, individuality, fear conditioning

Early life experience has a long-lasting influence on the development of neural systems implicated in emotion and cognition (for reviews, see Gordon & Hen, 2004; Zhang, Bagot, et al., 2006). In the rat, natural variations in maternal care, particularly licking, contribute to the development of stable individual differences in anxiety. Adult rats that had received relatively high levels of maternal licking during infancy display less anxiety-related behavior in response to aversive situations than less frequently licked rats. Thus, the former show decreases in shock-induced freezing, probe burying, acoustic startle, and decreased latencies to begin feeding in a novel environment, but increased exploration of a novel open field in comparison to the latter (Caldji et al., 1998; Francis, Diorio, Liu, & Meaney, 1999; Menard, Champagne, & Meaney, 2004, Menard & Hakvoort, 2007; Zhang et al., 2005). These behavioral differences are accompanied by alterations in physiological stress reactivity (Liu et al., 1997) and receptor systems in brain areas implicated in anxiety regulation (Caldji et al., 1998).

Behaviorally, ultrasonic vocalizations have been proposed as a useful measure of anxiety in infant and adult rats (for reviews, see Branchi, Santucci, & Alleva, 2001; Hofer, 1996; Sanchez, 2003). Rat pups emit calls in the ultrasonic range, often termed 40-kHz calls, in response to several distressing situations, like separation from litter and mother or when ambient temperature drops (for reviews, see Constantini & D'Amato, 2006; Hofer, 1996). Such isolation-induced calls serve as an index of pup anxiety, since calling behavior can be attenuated by anxiolytic compounds, and increased by anxiogenic ones (Gardner, 1985; Insel, Hill, & Mayor, 1986). Furthermore, Insel et al. (1986) showed that anxiolytics affect not only call number, but also acoustical call parameters, like amplitude and frequency. It was also found that animals bred for high rates of isolation-induced calling in infancy show more anxiety-related behavior in adulthood than animals bred for low call rates (Brunelli, 2005; Zimmerberg, Brunelli, Fluty, & Frye, 2005). These so called "distress calls" seem to play an important role in pup survival, since they can elicit maternal behavior. They induce maternal searching and pup retrieval (Allin & Banks, 1972; Brunelli, Shair, & Hofer, 1994; Hashimoto, Saito, Furudate, & Takahashi, 2001; Smotherman, Bell, Starzec, Elias, & Zachman, 1974), and shorten the response to transport litters from an endangered nest (Brewster & Leon, 1980). Besides, they elicit an increase in anogenital licking by the mother (Brouette-Lahlou, Vernet-Maury, & Vigouroux, 1992) and induce prolactin release in lactating females (Hashimoto et al., 2001; Terkel, Damassa, & Sawyer, 1979). Interestingly, Brudzynski, Kehoe, and Callahan (1999) postulated that not only call number is crucial for the stimulation of maternal behavior, but also certain acoustical call parameters, like frequency modulation.

Juvenile and adult rats produce low-frequency calls, often termed 22-kHz calls, during confrontation with predators (Blanchard, Blanchard, Agullana, & Weiss, 1991), submissive behavior during intermale fighting (Kaltwasser, 1990), or exposure to aversive stimuli, like startling noise (Kaltwasser, 1991), handling and touch (Brudzynski & Ociepa, 1992), air puffs (Knapp & Po-

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horecky, 1995), or electric shock (Borta, Wöhr, & Schwarting, 2006; De Vry, Benz, Schreiber, & Traber, 1993; Jelen, Soltysik, & Zagrodzka, 2003; Tonoue, Ashida, Makino, & Hata, 1986; Van der Poel & Miczek, 1991; Wöhr, Borta, & Schwarting, 2005). Such vocalizations are not only displayed during the actual aversive event, but may also occur in response to stimuli associated with such experiences (Borta et al., 2006; Wöhr et al., 2006). These 22-kHz calls are considered as part of the animal's defensive repertoire (Brudzynksi, 2001), since they are closely associated with the freezing response to actual or potential threat (Wöhr et al., 2005), and might serve as alarm calls for conspecifics (Blanchard et al., 1991). Therefore, it was assumed that they reflect a negative affective state akin to anxiety and depression (Jelen et al., 2003; Tonoue et al., 1986). Accordingly, it was found that call likelihood and number depend on the aversiveness of the situation (Wöhr et al., 2005), and individual disposition to show anxiety-related behavior when measured using an elevated plus maze test (Borta et al., 2006). Moreover, it was found that anxiolytic compounds can reduce calling behavior, whereas anxiogenic compounds increase it (Sanchez, 2003).

The first objective of the present study was to assess whether variations in maternal care are associated with individual differences in anxiety-related behavior already during early life. Maternal care was measured using several indices, namely licking, retrieval behavior, and maternal responsiveness to playback of pup calls. This latter test was chosen, since it gives the opportunity to test maternal behavior under exclusion of olfactory and tactile stimulation by pups. To test anxiety-related behavior in the infant rat, pups were isolated and their behavioral response to the separation from litter and mother measured. As an index of anxiety, isolation-induced infant calls were used.

The second objective of the study was to test whether infant indices of anxiety are related to anxiety-related behavior in adulthood. Here, two strategies were chosen. First, it was tested whether isolation-induced overt behavior or infant calling can be used as a predictor of anxiety-related behavior in adulthood, which was assessed in an activity box, an elevated plus maze, and a fear conditioning paradigm. Second, it was asked whether variations in maternal care had similar relationships with anxiety-related behavior during adulthood as compared to infancy.

Materials and Method

Animals and Housing

Fifty male Wistar rat pups of 10 litters and their 10 biological mothers (HsdCpb:WU, Harlan-Winkelmann, Borchen, Germany) served as subjects. Rat pups were delivered to the laboratory on pnd 3 together with their mothers. To avoid effects of litter size (Dimitsantos, Escorihuela, Fuentes, Armario, & Nadal, 2007), each litter consisted of five pups. Females and surplus males were removed from the nest. Each litter was housed with its mother on Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland) in a Macrolon type IV cage (size: 378 × 217 × 180 mm, plus high stainless steel covers) that permitted a clear view from all sides. Lab chow (Altromin, Lage, Germany) and water (0.0004% HCl solution) were available ad libitum. Animals were housed in an animal room with a 12:12 h light/dark cycle (lights on 7–19 h) where the environmental temperature was maintained between 23° and 29° Celsius (humidity: 32%–65%).

General Procedure

On pnd 3, the experimental animals (6 litters = 30 pups) were weighed and marked with a surgical marker (Codmen pen, Johnson & Johnson, New Brunswick, NJ) to identify them individually. A given pup was marked either on one of its limbs or on the abdomen, and marking was refreshed on pnd 4, 6, 8, 9, 10, 11, 13, 17. Another 20 pups, that is, 4 litters, served as controls for the early marking procedure. These controls remained undisturbed until pnd 11. From pnd 11 onward, both groups were treated identically.

On pnd 3–6, pup licking was measured daily for 6 h in experimental and control rats. These days of observations were chosen according to Champagne, Francis, Mar, and Meaney (2003), who showed that mothers differ in their licking behavior primarily between pnd 2–8.

On pnd 4, 6, 8, 9, and 10, retrieval behavior was monitored for 5 min in mothers of experimental rats. These test days were chosen according to Grota and Ader (1969a), who showed that retrieval behavior can be reliably induced by placing a rat pup outside the nest during the first two weeks after birth.

On pnd 11, all pups were isolated individually from nest and mother for 10 min. Overt behavior and 40-kHz call production was recorded. Pnd 11 was chosen, since (1) ultrasonic call production is rather independent from changes in temperature during the second week of life relatively to the first week, (2) high within-litter-variability in call emission was observed at this time-point, and (3) substantial evidence for intraindividual stability in call emission was provided (Brunelli, Keating, Hamilton, & Hofer, 1996; Brunelli, Vinocur, Soo-Hoo, & Hofer, 1997).

On pnd 21, pups were weaned. Immediately after weaning, the mothers' responses to playback of 40-kHz vocalizations were tested.

In adulthood, the animals were tested in an activity box to measure individual levels of locomotor activity on pnd 94 and 95, after 3 days of handling (each rat 5 min per day). Four days later, on pnd 99, animals were screened for anxiety-related behavior on an elevated plus maze. Finally, on pnd 121–123, a fear conditioning paradigm was conducted to test for 22-kHz vocalizations.

All behavioral tests were conducted between 9 and 17 h. Prior to each test, behavioral equipment was cleaned using a 0.1% acetic acid solution followed by drying.

Maternal Care

Licking Behavior

Continuous observations were made at regular times each day with two periods during the light phase of the light/dark cycle (9–12 h and 14–17 h). These time-periods were based on the finding that maternal behavior occurs more frequently during the light phase (Grota & Ader, 1969a). The numbers of anogenital and body lickings were scored. In experimental rats, they were scored individually, whereas in control rats the total amounts of anogenital and body lickings were scored irrespective of the individual pup.

Retrieval Behavior

Retrieval behavior was measured between 12 and 13 h. First, the mother was removed from the housing cage and isolated in a

Makrolon type III cage. Then, all five pups of a given litter were removed from the housing cage and markings were refreshed. After approximately 5 min of separation, pups were scattered over the floor of the housing cage and the mother was reintroduced. Latencies to retrieve the first and the last pup were measured. Observations ended when all pups were retrieved, or when 5 min had elapsed, that is, a ceiling score of 5 min was used when a mother did not retrieve all pups.

Search Behavior

Immediately after weaning, the mothers were exposed to acoustic stimuli on a radial maze of gray plastic with 8 arms (9.8 × 40.5 cm) extending radially from a central platform (diameter: 24 cm; for details, see Görisch & Schwarting, 2006). An ultrasonic speaker (ScanSpeak, Avisoft-Bioacoustics, Berlin, Germany), which had a frequency range of 1–120 kHz with a relatively flat frequency response (± 12 dB) between 15 and 65 kHz, was placed 20 cm away from the end of one arm.

Testing was performed under white light (approximately 30 lux in the center of the maze) in a testing room with no other rats present. A given mother was placed on the central platform of the radial maze, facing the arm opposite to the loudspeaker. After 10 min (termed habituation), noise (equally distributed between 20 and 120 kHz, about 55 dB) was presented for 1 min. After another 10 min without tone presentation (inter-stimulus-interval 1, ISI 1), a sine wave tone (40 kHz, about 55 dB) was presented for 1 min. Noise and 40-kHz sine wave tone were generated with SASLab Pro (version 4.2, Avisoft Bioacoustics, Berlin, Germany). After another 10 min (ISI 2), natural 40-kHz calls were presented for 1 min. A total of 161 calls were presented (total calling time: 16.61 s, call duration: 103 ± 5 ms, peak frequency: 42.52 ± 0.29 kHz, peak amplitude: about 55 dB, frequency modulation: 13.53 ± 0.26 kHz). These calls had been recorded from a male Wistar rat pup on pnd 11 during isolation (for setting and recording, see "Isolation" and "Recording and Analysis of Ultrasonic Vocalization," respectively). After two additional min without tone presentation (ISI 3), the test ended (total test duration: 35 min).

Behavior was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany), which was mounted 150 cm above the maze, and connected to a video recorder (Panasonic, CVC SQPB). Potential maternal ultrasonic calls were recorded by an Electret ultrasound microphone (Emkay FG-3629, Avisoft Bioacoustics) placed 20 cm away from the maze next to the loudspeaker.

Behavioral analysis was performed in two ways. A trained observer scored the videos for orienting responses during stimulus presentation, like head orientation (orienting movements restricted to the head) or body orientation (orienting movements of almost the entire body resulting in a stretched posture), and the time spent on the three arms proximal or distal from the ultrasonic speaker. Finally, the total distance traveled in cm was analyzed using an automated video tracking system (Ethovision, Noldus, Wageningen, The Netherlands).

Isolation

To induce 40-kHz vocalizations, pups were isolated from the mother and nest on pnd 11 for 10 min under room temperature (20.8 °C to 23.2 °C). Pups were removed individually from the nest at random and gently placed into an isolation box (23 × 28 × 18 cm) made of white and transparent plastic walls. The roof and one wall were made of transparent plastic to allow video observation during the test. The isolation box was placed in a sound attenuating isolation cubicle (51 × 71 × 51 cm; Coulbourn Instruments, Allentown, PA) equipped with 2 white-light LED spots (63 lux, Conrad Electronic GmbH, Hirschau, Germany) and a black/white CCD camera (Conrad Electronic GmbH) connected to a DVD recorder (DVR-3100 S, Pioneer, Willich, Germany). Ultrasonic vocalization was recorded using an Electret ultrasound microphone (Emkay FG-3629, Avisoft Bioacoustics) placed in the roof of the box, 12 cm above the floor.

Behavioral activity was measured as rearing (number of rears with at least one paw off the floor), head-raising (number of times in which the head was raised above shoulder level), pivoting (number of 360° rotations), and locomotion. To measure locomotion, the isolation box was divided into four virtual squares and a count was obtained when all four paws crossed a grid line. In addition, myoclonic twitches were scored. A twitch was defined as a phasic, rapid, and independent movement of a forelimb, hind limb, or the tail. Finally, the duration spent grooming was measured in s. All behavioral measures were analyzed from DVD by an experienced observer. 40-kHz vocalizations were also measured (for details, see "Recording and Analysis of Ultrasonic Vocalization").

Animals of the same litter were never tested successively, that is, at least 30 min were allowed to elapse between pups of the same litter in order to avoid maternal arousal. Besides, this procedure guaranteed that context effects, like a weak increase in ambient temperature throughout testing, were equally distributed over all litters.

After isolation, animals were weighed and marked, that is, body marks were renewed in experimental rats, while control rats were marked the first time.

Tests in Adult Rats

Activity Box

A small open field (acrylic; 40 × 40 × 40 cm) monitored by an automated activity monitoring system (Tru Scan, Photobeam Sensor-E63-22, Coulbourn Instruments, Allentown, PA) was used, and the number of rears and locomotor activity (distance traveled in cm) scored. The time spent in the center was also measured (for details, see Borta & Schwarting, 2005). Behavior was tested under red light (28 lux) for 10 min on two consecutive days.

Elevated Plus Maze

The elevated plus-maze described in Borta et al. (2006) was used. Each animal was tested once under white light (30 lux) for 5 min. It was placed in the center of the plus maze, facing one of the open arms. The following behavioral measures were analyzed from videotapes: (1) the number of entries into open or closed arms (an entry was scored when all four paws crossed into the arm), (2) the time spent on open or closed arms, and (3) the duration of risk assessment, which was scored when the animal's body was in a stretched position between an open and a closed arm (for details, see Schwarting, Jegan, & Wöhr, 2007).

Fear Conditioning

A 3-day procedure was used here. On the first day (termed habituation day), each rat was placed in the shock chamber for 11 min to measure baseline behavior and possible vocalization. After 24 h (termed conditioning day), it was placed again into the shock chamber for 11 min. After an initial phase of 3 min where no tone or shock was given, the rat was exposed to six tone/shock pairings, each followed by an ISI of 60 s. As the conditioned stimulus (CS), a 3-kHz sine wave tone (generated with: SASLab Pro, version 4.2, Avisoft Bioacoustics) was presented for 20 s, and as the unconditioned stimulus (UCS) a 0.5 mA scrambled shock (52 Hz, peak-to-peak amplitude 120 V; Med Associates, Stand alone shocker) was used. This shock was administered during the last 500 ms of the tone. On the third day (termed testing day), the rat was again placed into the shock chamber for 11 min. After an initial phase of 3 min, the tone (but no shock) was presented six times for 20 s each. The test was performed under bright white light (about 200 lux) in a standard shock chamber described in Wöhr et al. (2005).

The duration of immobility (lack of all somatic mobility except respiratory activity) was scored from DVD by an experienced observer. 22-kHz vocalizations were also measured (for details, see "Recording and Analysis of Ultrasonic Vocalization").

Recording and Analysis of Ultrasonic Vocalization

An Electret ultrasound microphone (Emkay FG-3629, Avisoft Bioacoustics) sensitive to frequencies of 10–120 kHz with a flat frequency response between 15 and 30 kHz (± 6 dB) and between 40 and 70 kHz (± 12 dB) was used. It was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft Recorder (version 2.7; Avisoft Bioacoustics) and were recorded with a sampling rate of 214,285 Hz in16 bit format.

For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution.

Forty-kHz call detection was provided by an automatic threshold-based algorithm (threshold: −50 dB) and a hold-time mechanism (hold time: 10 ms). A lower-cut-off-frequency of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of call detection was verified by an experienced user (detection rate: 99.2%, false alarm rate: 0.0%). When necessary, missed calls were marked by hand to be included in the automatic parameter analysis. Various parameters, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element, were determined automatically. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. The extent of frequency modulation, that is, the difference between the lowest and the highest peak frequency within each call was also measured automatically. Temporal parameters determined included latency to call, call duration, total calling time, and the duration of intervals between subsequent calls. Finally, the total number of calls emitted was measured.

22-kHz call detection was provided by an automatic threshold-based algorithm (threshold: −40 dB) and a hold-time mechanism (hold time: 20 ms). A lower-cut-off-frequency of 18 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. An experienced user checked the accuracy of call detection and obtained a 100% concordance between automatic and behavioral detection. Thereafter, the same call parameters were determined as for the 40-kHz calls. Based on the duration of the interval between two calls, calls were divided into those starting a bout versus those within a bout. A bout was defined as a call, or a number of calls, which was separated from other calls by intervals longer than 320 ms, according to Van der Poel (1991; for details, see Wöhr et al., 2005). To describe the temporal patterning of call production, the numbers of bouts and bout-length were examined.

Statistical Analysis

To determine bout-length, call duration, peak frequency, peak amplitude, and the extent of frequency modulation, the mean of each call parameter served as the statistical unit in each subject. To test whether control and experimental rats differ in the level of maternal care experienced, an analysis of variance (ANOVA) for repeated measurements was used. Other comparisons between control and experimental rats were done by using unpaired *t* tests, or when a normal distribution was not given (based on Kolomogorov-Smirnov test), by using Mann-Whitney *U* tests. Experimental rats were ranked according to the number of lickings experienced. The upper and lower quartiles of the population were assigned to the HL (highly licked) and LL (low licked) subgroup, respectively. Moreover, using a median split, experimental rats were also divided into animals reared by mothers which retrieved their pups fast or slowly, and animals reared by mothers which spent a long time on the proximal arms when 40-kHz calls were presented or mothers which spent a short time on such arms. Unpaired *t* tests or Mann-Whitney *U* tests were used for comparisons between these groups. An ANOVA for repeated measurements was used to test whether HL and LL differ in the time course of ultrasonic calling and whether both subgroups differ in their overt behavior in the activity box on two consecutive days. An ANOVA for repeated measurements was also used to test whether maternal locomotor activity differs over time during the test for playback-induced search behavior. The time spent on proximal or distal arms on the radial maze was also compared with paired *t* tests. Finally, an ANOVA was used to test whether the position of the markings affected maternal licking behavior. Pearson's correlation coefficient was used to test whether overt behavioral parameters were related to each other, or to ultrasonic calling, or whether ultrasonic calling was correlated between different tests. When a normal distribution was not given, the nonparametric Spearman's correlation coefficient was calculated. All correlation coefficients are based on experimental rats only. A principal component analysis with varimax rotation using the Kaiser criterion (eigenvalues >1) was calculated to examine patterns of relationships among overt behavioral variables measured in isolation. The exact *p* values of 2-tailed testing were taken as measures of effect.

Results

Maternal Care

Licking Behavior

During the total observational period of maternal licking (i.e., 24 h), mothers licked their pups 148 ± 13 times (mean ± *SEM*, range = 84–236), meaning that each pup was licked on average 1.25 times per hour. Predominately, anogenital licking occurred (113 ± 10, range = 72–176), whereas body licking occurred less often (35 ± 6, range = 11–61). Since both measures were highly correlated ($\rho = .943$, $p = .005$) they were pooled to one measure. This total amount of licking increased significantly over days, $F(3,6) = 6.289$, $p = .028$, but did not differ between experimental and control pups, $F(1,8) = 2.683$, $p = .140$, and no interaction between group and observation day was observed, $F(3,6) = 2.613$, $p = .146$; see Table 1. Furthermore, in experimental pups anogenital licking and body licking occurred independent from the position of markings, $F(2,29) = .341$, $p = .714$ and $F(2,29) = .572$, $p = .571$, respectively.

Based on the number of total lickings, experimental pups were divided into HL (i.e., upper quartile of the population; $n = 8$) and LL (i.e., lower quartile of the population; $n = 10$) rats. HL pups were licked 34.6 ± 1.5 times, compared to 16.2 ± 0.8 times in LL pups ($T_{16} = -11.865$, $p < .001$). Since licking was characterized by a high intralitter variability (smallest range = 15–21; highest range = 20–40), pups of 2 out of 6 litters were represented in both subgroups, that is, HL and LL pups.

Retrieval Behavior

The likelihood to retrieve pups was dependent on pup age, that is 5 out of 6 mothers retrieved all of their pups on pnd 4, as compared to only 2 mothers on pnd 10. Over all the 5 days of observation, mothers started to retrieve pups after 95.1 ± 41.9 s and finished retrieving after 166.7 ± 41.1 s. Since both measures were highly correlated ($r = 0.838$, $p = .037$), only the latency to retrieve the last pup will be used further.

Based on this latency, experimental pups were divided into those reared by rats which retrieved them slowly, namely after 244.0 ± 28.6 s, or fast, namely after 104.3 ± 33.3 s ($T_4 = 3.180$, $p = .034$; $n = 15$ in each group).

Search Behavior

Playback of all three acoustic stimuli were clearly audible to the mothers, since they induced orienting responses (noise: 10/10 animals, 40-kHz tone: 6/10, 40-kHz calls: 9/10). Interestingly, playback-induced locomotor activation was stimulus-dependent (see Figure 1). Throughout the testing period, the distance traveled during the minute preceding each stimulus presentation declined successively, $F(2,8) = 4.533$, $p = .048$, indicating habituation to the general testing situation. In contrast, declines during stimulus presentations were not significant, $F(2,8 = 3.433)$, $p = .084$). Subsequent paired t tests revealed that the distance traveled was significantly reduced during the minute preceding 40-kHz call presentation in comparison to that preceding noise ($T_9 = 2.838$, $p = .019$). Such a reduction was not observed during stimulus presentation ($T_9 = 1.019$, $p = .335$), indicating that mothers explored the maze more during the presentation of 40-kHz calls in comparison to the minute before ($T_9 = -2.086$, $p = .067$). No such effects were observed in case of noise ($T_9 = 1.020$, $p = .334$) or tone presentation ($T_9 = .667$, $p = .522$).

When analyzing this call-induced locomotor activation in more detail, it was found that it was stimulus-directed (see Figure 1), since the mothers showed a clear preference for the three arms proximal to the ultrasonic speaker during 40-kHz call presentation as compared to the distal ones ($T_9 = 3.165$, $p = .011$). Such a preference was not observed during noise ($T_9 = .934$, $p = .375$), 40-kHz tone ($T_9 = .690$, $p = .508$), or ISIs (all p values $>.100$). However, it has to be mentioned that animals initially (min 1–10) preferred the distal arms relative to the proximal arms ($T_9 = -4.945$, $p = .001$). Furthermore, mothers of experimental and control pups did not differ significantly in their time spent on the proximal arm during playback of 40-kHz calls ($T_8 = .159$, $p = 877$). Finally, it is worth to note that dams did not emit ultrasonic vocalizations during this test.

Based on the time spent by mothers in the proximal arms during presentation of 40-kHz calls, experimental pups were divided into those reared by mothers which spent little (3.1 ± 3.1 s) or much (44.3 ± 9.2 s) time on the proximal arms ($T_4 = -4.261$, $p = .013$; $n = 15$ in each group).

Relations Between Maternal Behaviors

Licking behavior, the latency to retrieve the last pup, and the time spent on the proximal arms were not significantly correlated to each other (all p values $>.100$). When pups reared by mothers with low proximal arm time were compared with pups reared by mothers with high proximal arm time, it was found that the latter pups were licked more often (20.3 ± 1.5 and 26.7 ± 2.2, respectively, $T_{25.324} = -2.398$, $p = .024$). Such a difference was not observed when comparing pups from mothers which had retrieved them slowly (21.7 ± 1.6) or fast (25.3 ± 2.4; $T_{24.398} = -1.263$, $p = .219$).

Table 1
Maternal Care: Total Licking

	Total	pnd 3	Pnd 4	pnd 5	pnd 6
Experimental rats	26.37 ± 2.89	6.03 ± 0.78	6.70 ± 1.01	6.30 ± 0.97	7.33 ± 0.74
HL	34.63 ± 1.45	6.88 ± 0.95	9.25 ± 1.08	8.50 ± 0.71	10.00 ± 0.91
LL	16.20 ± 0.77	4.00 ± 0.49	3.50 ± 0.50	4.10 ± 0.48	4.60 ± 0.54
Control rats	34.55 ± 4.36	6.55 ± 1.19	7.55 ± 1.05	10.65 ± 2.07	9.80 ± 0.67

Note. HL = highly licked; LL = low licked. Values reflect means ± *SEM* (Experimental rats: $n = 30$, HL: $n = 8$, LL: $n = 10$, control rats: $n = 20$). Given are average values per pup.

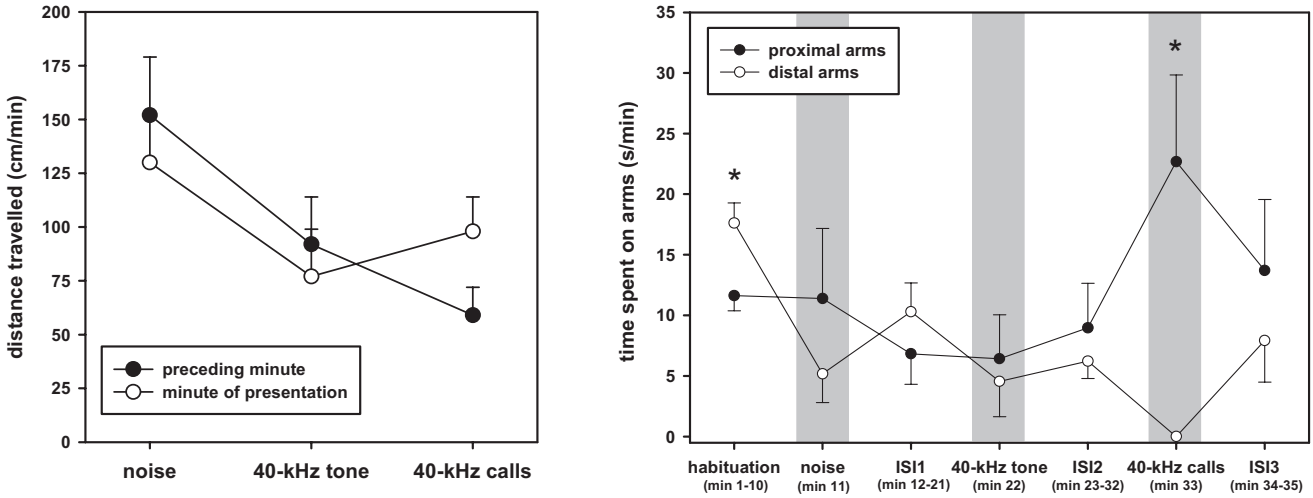

Figure 1. Time courses of maternal activity during the test for search behavior. On the left, the distance traveled on the radial maze is given in cm for the minutes of presentations of noise, 40-kHz tone, and 40-kHz calls (open symbols) as also for the respective minutes before (filled symbols). On the right, the time spent on the proximal (filled symbols) or distal (open symbols) arms from the loudspeaker is given for habituation, inter-stimulus-intervals, and playback of acoustic stimuli, that is, noise, 40-kHz tone, and 40-kHz calls. Significant differences between the time spent on the proximal and on the distal arms are marked with asterisks: $*p < .05$. Values reflect means ± *SEM* per minute.

Isolation

Overt Behavior

In response to separation from mother and litter, rat pups showed marked behavioral activation. All animals showed head raising during isolation (27.0 ± 2.7, range = 2–84). Furthermore, 49 out of 50 pups performed at least one 360°-rotation (4.6 ± 0.4, range = 0–15). Similar results were obtained for the number of squares crossed, since locomotor activity was almost totally restricted to such pivoting. On the average, animals crossed 5.6 ± 0.8 squares (range = 0–29, 49 of 50 animals showed square crosses). Rearing behavior occurred less often, that is, only 30 of 50 animals showed it, solely in the form of on-wall rears (3.3 ± 0.8, range = 0–34). Twitching was shown by 42 of 50 pups (7.6 ± 1.0, range = 0–26), and all animals showed at least some grooming (41.4 ± 3.1 s, range = 1–102 s).

Body weight on this testing day was not significantly correlated with any overt behavior (all p values >.100). Apart from the observation that experimental rats tended to show more head-raisings than control rats ($U = 209.50$, $p = .076$), these groups did not differ (all p values >.100).

Ultrasonic Vocalization

Behavioral activation was accompanied by the emission of ultrasonic vocalizations. All 50 pups emitted ultrasonic calls and started to call almost immediately on separation (2.8 ± 0.4 s, range = 0.4–13.7 s). On the average, 846.2 ± 60.7 calls were emitted and animals spent 105.9 ± 7.3 s calling. However, call emission was characterized by huge individual differences, that is, the animal with the lowest call rate emitted only 71 calls, whereas that with the highest call rate emitted 2534 calls. Similar individual differences were observed in total calling time (range = 7.7–201.1 s). The average duration of the intervals between two calls was 557 ± 9 ms (range = 11 ms to 133.82 s). As shown in Figure 2, the distribution of these intervals had 3 peaks, indicating a bout structure, with a first peak at 11 ms, a second at 87 ms and a third at 233 ms. Based on the duration of intervals, calls were divided into calls starting a bout versus those within a bout. A bout was defined as a call, or a number of calls, separated from other calls by intervals longer than 196 ms, since the least frequent duration of an interval between the second and the third peak was 196 ms. Pups emitted 347.1 ± 16.3 bouts (range = 43–553), which consisted of 2.3 ± 0.1 calls (range = 1.5–5.2). These calls had a peak frequency of 44.0 ± 0.6 kHz (range = 37.1–55.0 kHz) and were displayed with 73.3 ± 0.7 dB (range = 57.0–80.9 dB). The extent of frequency modulation was 9.7 ± 0.8 kHz (range = 3.2–33.6 kHz).

Body weight was negatively correlated with the number of calls emitted within one bout ($r = -0.556$, $p = .001$), and tended to correlate with call number ($r = -0.321$, $p = .083$; all other p values >.100). Regarding call emission, experimental and control rats did not differ (all p values >.100).

Relations Between Overt Behavior and Ultrasonic Vocalization

Overt behavior and ultrasonic calling were highly correlated (see Figure 3), especially in case of pivoting. The number of such 360° rotations was positively correlated with call number ($\rho = .640$, $p < .001$; see Figure 4), bout number ($\rho = .678$, $p < .001$), total calling time ($\rho = .551$, $p = .002$), and tended to correlate with bout length ($\rho = .338$, $p = .067$; all other p values >.100). The number of squares crossed was also positively correlated with calling behavior (number of calls: $\rho = .539$, $p = .002$, bout number: $\rho = .617$, $p < .001$, and total calling time: $\rho = .462$, $p = .010$, peak frequency: $\rho = -.419$,

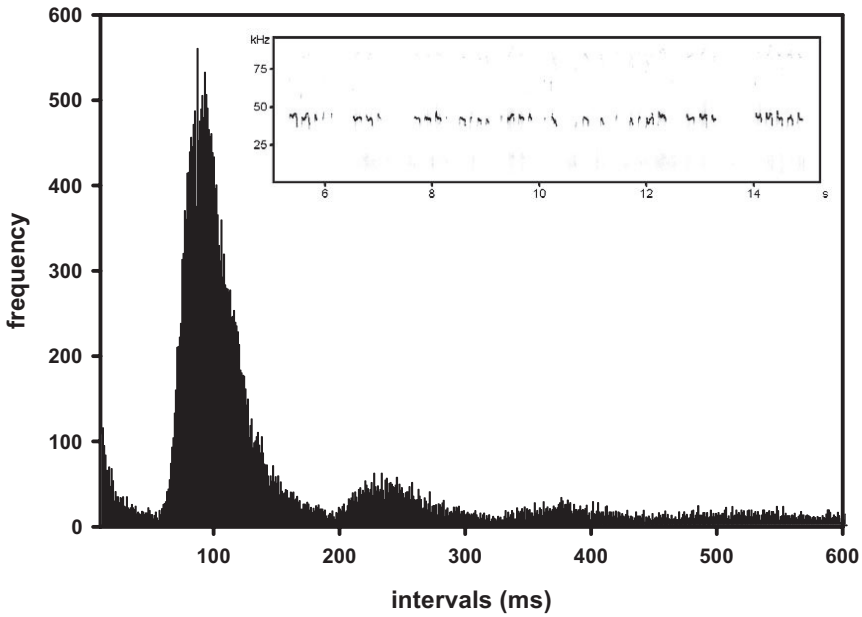

Figure 2. Histogram depicting the distribution of intervals between two subsequent calls. A small portion of the data is not illustrated to keep the abscissa of the distribution within a reasonable size for presentation. The embedded graph depicts a spectrogram of an exemplary sequence of isolation-induced ultrasonic vocalizations showing the bout structure.

$p = .021$, peak amplitude: $\rho = .370$, $p = .044$; all other p values $>.100$). Also, head-raising was positively correlated with bout number ($\rho = .440$, $p = .015$), and tended to correlate with call number ($\rho = .333$, $p = .072$) and total calling time ($\rho = .345$, $p = .062$; all other p values $>.100$). Rearing was only weakly associated with calling, that is, apart from a negative correlation with the latency to the first call uttered ($\rho = -.440$, $p = .015$), only a trend for a correlation with call duration was found ($\rho = -.353$, $p = .055$; all other p values $>.100$) Twitching and grooming were not significantly correlated with call emission (all p values $>.100$). In short, call emission was positively correlated with behavioral measures which reflect locomotor activation, but not with twitching and grooming. Interestingly, a factor analysis revealed two factors of overt behavior with

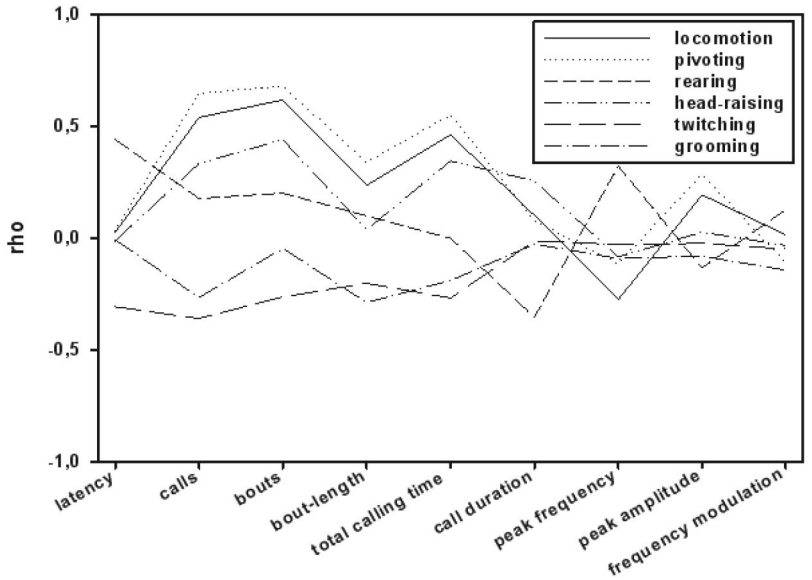

Figure 3. Graph depicting the correlation coefficients (Spearmans ρ) between call parameters and overt behavioral parameters. Each line reflects a different behavioral parameter.

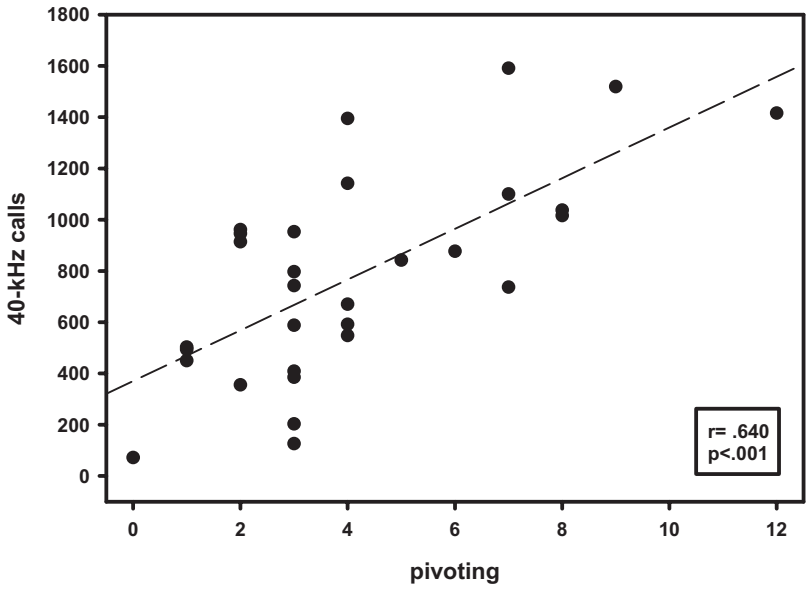

Figure 4. Scatter plot depicting individual relationship between ultrasonic vocalization (40-kHz alls; number) and overt behavior (pivoting; number) displayed during the 10 min of isolation.

eigenvalues above 1, namely locomotor activation and grooming (see Figure 5 and Table 2), reflecting this pattern.

Comparison Between HL and LL Rats

HL and LL pups did not differ in twitching, rearing, head-raising, pivoting and the numbers of squares crossed (all p values $>.100$), though HL pups spent more time grooming than LL pups ($U = 45.00$, $p = .043$; see Table 3). Accordingly, only total licking and grooming were positively correlated ($\rho = .423$, $p = .020$; all other p values $>.100$).

In contrast to overt behavior, ultrasonic call production was strongly affected by the amount of licking experienced (see Table 4). HL rats emitted 462.0 ± 101.0 40-kHz calls during isolation, whereas LL rats emitted twice as many (1026.7 ± 119.4, $T_{16} = 3.496$, $p = .003$; see Figure 6). In addition, HL rats emitted fewer and shorter bouts than LL rats ($T_{16} = 2.781$, $p = .013$ and $T_{16} = 3.529$, $p = .003$, respectively), and showed a trend for shorter call durations ($T_{16} = 1.856$, $p = .082$), which resulted in an overall less total calling time in HL rats ($T_{16} = 3.513$, $p = .003$). Also, calls emitted by HL rats were lower in amplitude ($T_{16} = 2.126$, $p = .049$), but more frequency modulated than those emitted by LL rats ($T_{16} = -2.219$, $p = .041$). No difference was observed for latency to the first call uttered ($T_{8.807} = -1.714$, $p = .121$) and peak frequency ($T_{16} = -1.387$, $p = .185$).

A temporal analysis (see Figure 7) revealed that the difference in call number between HL and LL rats was evident during each min of isolation, $F(1,16) = 12.219$, $p = .003$, all p values of subsequent t tests $<.050$. Besides this, there was a clear decline in call rates over time in both groups, $F(9,8) = 6.806$, $p < .001$, indicating within-session habituation. Interestingly, the gradual decrease in call number was accompanied by a gradual increase in call duration, $F(9,8) = 5.329$, $p < .001$, resulting in an inverted U-shaped development of the time spent calling, $F(9,8) = 2.366$, $p = .016$. The difference between HL and LL rats in total calling time was observable within each min, $F(1,16) = 12.339$, $p = .003$, all p values of subsequent t tests $<.050$. Additional changes over time were observed for peak frequency, $F(9,8) = 4.213$, $p < .001$, peak amplitude, $F(9,8) = 4.021$, $p < .001$, and frequency modulation, $F(9,8) = 2.702$, $p = .007$. However, in case of call duration, peak frequency, peak amplitude and frequency modulation, groups

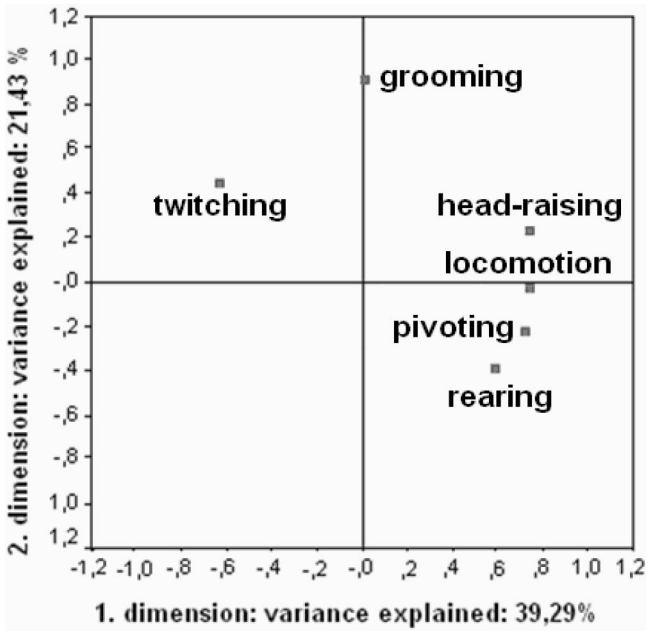

Figure 5. Illustration of factor loadings in a two-dimensional plane with two independent components after rotation.

Table 2
Isolation: Factor Analysis of Overt Behavior

	1. Dimension	2. Dimension
Locomotion	.744	−.023
Pivoting	.714	−.221
Rearing	.586	−.385
Head raising	.744	.236
Twitching	−.630	.451
Grooming	.009	.910
Variance explained	39.29%	21.43%

Note. Values in columns reflect factor loadings, which express the association of each variable to the dimension. Variance explained gives the percentage of variance in the entire data set accounted for by each dimension.

did not differ (all p values $>.100$). Interactions between group and time were not observed (all p values $>.100$), except for frequency modulation, $F(9,8) = 3.075$, $p = .003$, due to stronger frequency modulation of calls emitted by HL rats within the first two min in comparison to LL rats (min 1: $T_{16} = -.2982$, $p = .009$; min 2: $T_{16} = -2.710$, $p = .015$; all other p values $>.100$).

A correlational analysis supported the previous differences, since it showed negative correlations between pup calling behavior and maternal licking: number of calls emitted ($r = -0.534$, $p = .002$; see Figure 8), bout number ($r = -0.448$, $p = .013$), bout-length ($r = -0.463$, $p = .010$), total calling time ($r = -0.548$, $p = .002$), and call duration ($r = -0.354$, $p = .055$). However, total licking was not significantly correlated with the latency to call, peak frequency, peak amplitude nor frequency modulation (all p values $>.100$).

Comparisons With Other Maternal Behaviors

Call rates did not differ between pups of mothers which had retrieved them slowly or fast (790.1 ± 99.4 and 768.7 ± 109.7, respectively; $T_{28} = .144$, $p = .886$). When comparing pups of mothers which had spent a long or low time on the proximal arms of the radial maze in response to pup calls, evidence for a negative relationship between maternal care and isolation-induced ultrasonic calling in rat pups was obtained. Thus, pups from mothers with low proximal arm time emitted 936.7 ± 103.6 calls, whereas pups from mothers with high proximal arm time emitted only 622.1 ± 87.6 calls ($T_{28} = 2.319$, $p = .028$).

Table 3
Isolation: Behavioral Profile of HL and LL Pups

	HL	LL	p
Locomotion (n)	4.50 ± 1.78	5.30 ± 1.13	.416
Pivoting (n)	3.25 ± 0.84	4.10 ± 0.75	.368
Rearing (n)	2.25 ± 0.73	4.80 ± 3.27	.466
Head-raising (n)	20.38 ± 6.97	32.00 ± 8.27	.891
Twitching (n)	10.25 ± 1.64	8.00 ± 2.57	.210
Grooming (s)	50.52 ± 8.15	33.13 ± 5.72	.043

Note. HL = highly licked; LL = low licked. Values reflect means ± *SEM* (HL: $n = 8$, LL: $n = 10$).

Table 4
Isolation: Ultrasonic Vocalization in HL and LL Pups

	HL	LL	p
Latency (s)	3.24 ± 1.02	1.39 ± 0.37	.121
Calls (n)	462.00 ± 100.98	1026.70 ± 119.48	.003
Bouts (n)	234.50 ± 52.59	398.60 ± 32.09	.013
Bout-length (n)	1.93 ± 0.08	2.53 ± 0.14	.003
Total calling time (s)	51.25 ± 15.51	126.99 ± 14.76	.003
Call duration (ms)	102.80 ± 9.5	124.80 ± 7.49	.082
Peak frequency (kHz)	46.46 ± 0.89	44.33 ± 1.16	.185
Peak amplitude (HB)	68.74 ± 1.17	74.07 ± 2.03	.049
Frequency modulation (kHz)	14.25 ± 2.65	8.04 ± 1.35	.041

Note. HL = highly licked; LL = low licked. Values reflect means ± *SEM* (HL: $n = 8$, LL: $n = 10$).

Tests in Adult Rats

Survival Rates

Four control animals died before reaching adulthood. One experimental rat died shortly after the adult test in the activity box. Another experimental rat was excluded from the analysis of fear conditioning, since the first shock was not delivered.

Activity Box

In general, animals responded to the novel situation with exploratory activity, which declined from the first to the second test (not shown in detail). Experimental and control rats did not differ in any behavioral parameter and no interaction between group and test day was observed (all p values $>.100$).

On the two days of testing, there were no general differences between HL and LL rats (all p values $>.100$). Over days, a reduction of behavioral activity became evident (distance traveled:

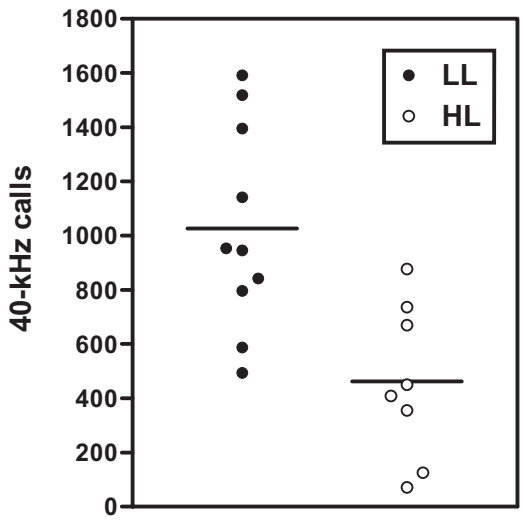

Figure 6. Column graph depicting individual levels of ultrasonic vocalization (40-kHz calls; number) in highly licked (HL: $n = 8$) and rarely licked animals (LL: $n = 10$).

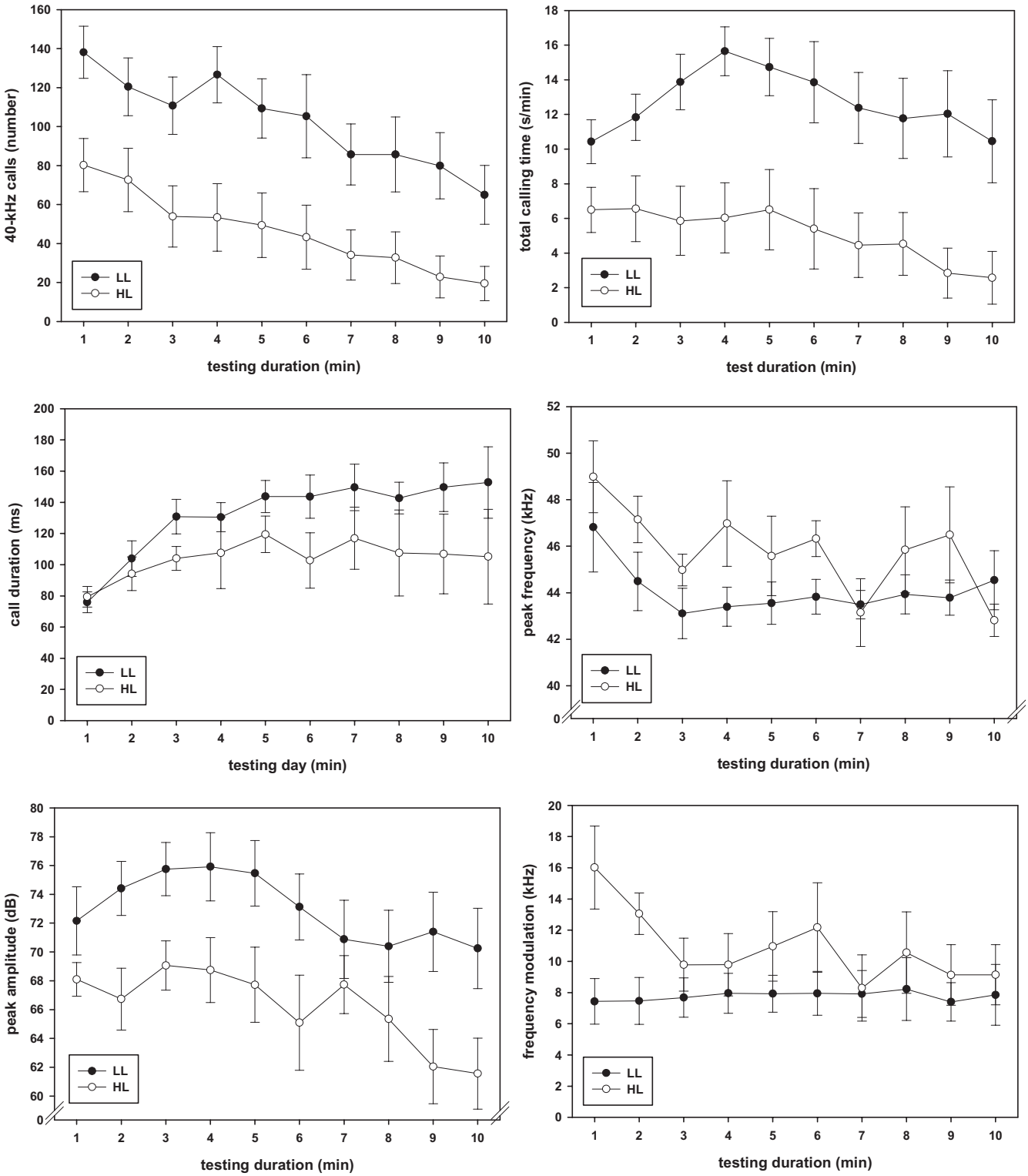

Figure 7. Time courses of ultrasonic vocalization per minute in HL ($n = 8$; open symbols) and LL rats ($n = 10$; filled symbols). Given are means ± *SEM* for 40-kHz call number, total calling time in s, call duration in ms, peak frequency in kHz, peak amplitude in dB, and frequency modulation in kHz.

$F(1,16) = 69.345$, $p < .001$; rearing: $F(1,16) = 115.289$, $p < .001$), while the time spent by the animals in the center was similar on both days, $F(1,16) = 2.359$, $p = .144$. Interactions between group and test day were not observed, apart from a trend for a more pronounced decline of rearing activity in HL relative to LL rats, $F(1,16) = 3.229$, $p = .091$; all other p values $>.100$; not shown in detail.

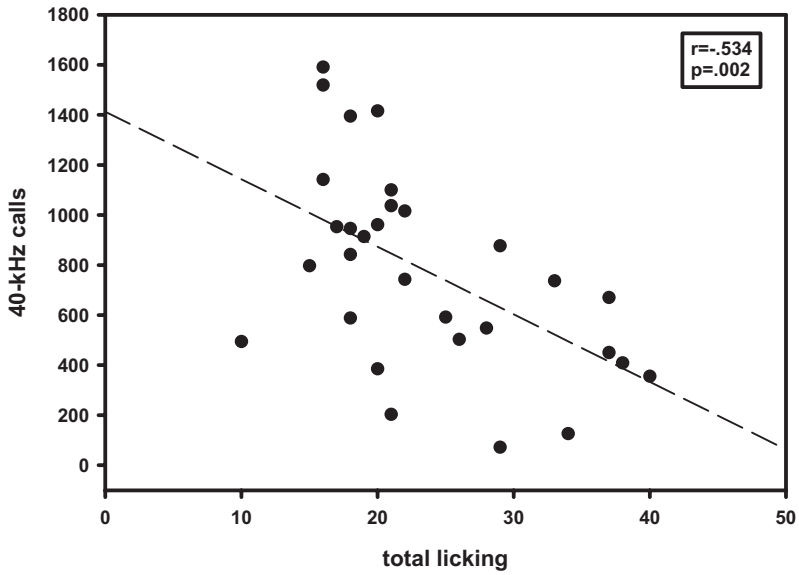

Figure 8. Scatter plot depicting individual relationship between ultrasonic vocalization (40-kHz calls; number) displayed during the 10 min of isolation and maternal care (total licking; number of anogenital and body licking during the total observational period, i.e., 24 h).

Correlational analyses yielded that only rearing activity on the second test was related to total pup licking ($r = -0.333$, $p = .072$; all other p values $>.100$). Infant ultrasonic calling, that is, call number and total calling time, was negatively associated with rearing activity during the first ($r = -0.358$, $p = .052$ and $r = -0.390$, $p = .033$, respectively; all other p values $>.100$), but not the second test (all p values $>.100$).

Also, infant overt behavior was associated with adult overt behavior. Thus, locomotion during isolation was negatively correlated with the distance moved in the activity box ($\rho = -.446$, $p = .013$) and the number of rears ($\rho = -.594$, $p = .001$) during the first test, and tended to correlate with the number of rears in the second ($\rho = -.316$, $p = .089$; all other p values $>.100$). Furthermore, the number of pivots tended to correlate with the number of rears on the first day ($\rho = -.358$, $p = .052$; all other p values $>.100$). Finally, pup grooming tended to correlate with the time spent in the center of the activity box during the first test ($\rho = -.350$, $p = .058$; all other p values $>.100$). Pup rearing, head-raising and twitching were not related to behavior in the activity box (all p values $>.100$).

Elevated Plus Maze

In general, the rats spent most of their time in the closed arms and entered them more often than the open ones (not shown in detail). The behavioral profile of experimental and control rats did not differ in any measure (all p values $>.100$).

Except for a trend for less open arm entries in HL rats ($T_{16} = 2.039$, $p = .058$), HL and LL rats did not differ in the plus maze (all p values $>.100$; see Table 5). Also, the number of lickings experienced as pups was not significantly correlated with any behavioral parameter (all p values $>.100$). Infant ultrasonic calling, that is, call number and total calling time, was not significantly correlated with plus maze behavior (all p values $>.100$), and infant overt behavior was only weakly associated with plus maze behavior, since grooming tended to correlate with risk assessment ($\rho = -.361$, $p = .055$; all other p values $>.100$).

Fear Conditioning

Overt behavior. During shock delivery on the conditioning day (min 4–11), the animals displayed short bursts of activation, with startle movements, flinches and running (not measured in detail). With repeated shock delivery, the predominant response was immobility. When reexposed to the context on the subsequent testing day (min 1–3), the level of immobility increased as compared to the initial 3 min periods of the habituation day and the conditioning day, indicating conditioned fear evoked by the context ($T_{27} = -13.794$, $p < .001$ and $T_{27} = -13.003$, $p < .001$, respectively). This enhanced immobility was also observed during the subsequent period of tone presentation (min 4–11), indicating conditioned fear evoked by context and CS ($T_{27} = -15.085$, $p < .001$ and $T_{27} = -5.457$, $p < .001$, respectively).

On the conditioning day, control and experimental rats did not differ behaviorally (all p values $>.100$). However, when reexposed to the context on the subsequent day (min 1 – 3), experimental rats tended to be longer immobile than control rats ($T_{23.069} = 2.014$, $p = .056$), but during min 4–11, there was no group difference ($T_{42} = -1.148$, $p = .257$).

Ultrasonic vocalizations. On the habituation day, only one rat emitted 22-kHz calls, and no rat vocalized during the initial 3 min on the conditioning day. During subsequent shock delivery, 22-kHz ultrasonic vocalizations were detected in 35 of 44 animals. On the average, 92.7 ± 12.3 calls were emitted (range = 0–279). When reexposed to the context on the subsequent testing day (min 1–3), none of the 44 animals emitted 22-kHz calls. However, when CS presentation started (min 4–11), 28 out of 44 animals began to

Table 5
Elevated Plus Maze: Behavioral Profile of HL and LL Rats

	HL	LL	*p*
Closed arm time	204.57 ± 22.69	191.89 ± 15.23	.638
Open arm time	63.94 ± 18.92	77.58 ± 14.40	.567
Closed arm entries	7.88 ± 0.55	8.10 ± 0.87	.840
Open arm entries	2.88 ± 0.61	4.70 ± 0.63	.058
Risk assessement	58.63 ± 10.62	43.29 ± 4.41	.213

Note. HL = highly licked; LL = low licked.Values reflect means ± *SEM* (HL: $n = 8$, LL: $n = 10$).

vocalize. On the average, 60.1 ± 12.3 calls were emitted (range = 0–267).

When comparing calling behavior in experimental and control rats on the conditioning day, only trends for differences were observed for bout number ($T_{41.986} = 1.932$, $p = .060$) and peak amplitude ($T_{33} = -1.953$, $p = .059$; all other p values $>.100$). On the testing day, the only difference between both groups was a reduced peak frequency in control rats ($T_{24} = 2.435$, $p = .023$; all other p values $>.100$).

Relations between overt behavior and ultrasonic calling. The number of 22-kHz calls emitted was positively correlated with immobility during min 4–11 on the conditioning day ($r = .681$, $p < .001$; see Figure 9) and on the testing day ($r = 0.385$, $p = .043$; see Figure 9). Similar results were obtained when correlating total calling time and immobility (conditioning day: $r = 0.694$, $p < .001$; testing day: $r = 0.387$, $p = .042$).

Comparison Between HL and LL Rats. Before shock delivery (min 1–3), immobility of HL and LL rats was similar ($T_{16} = -.926$, $p = .368$; see Table 6). During shock delivery (min 4–11), however, HL rats were immobile for a longer period than LL rats ($T_{16} = -2.168$, $p = .046$). Accordingly, correlational analyses yielded that the total number of lickings experienced as pups tended to be positively correlated with the time spent immobile during min 4–11 ($r = .324$, $p = .092$), but not during min 1–3 ($r = .162$, $p = .411$). On the testing day, HL and LL rats showed no behavioral differences (all p values $>.100$). Also, no significant correlations were obtained between total licking and behavioral measures (all p values $>.100$).

In addition, HL and LL rats differed in adult call production (see Table 7). First, 7 out of 8 HL rats (87.5%), but only 6 out of 10 LL rats (60.0%) vocalized when shocks were delivered on the conditioning day. Most importantly, HL rats emitted 121.6 ± 31.5 22-kHz calls, whereas LL rats emitted only 43.1 ± 17.24 calls ($T_{16} = -2.310$, $p = .035$). This effect is primarily based on the emission of more bouts by HL rats in comparison to LL rats ($T_{16} = -2.578$, $p = .020$), since bout-length did not differ between groups ($T_{16} = -.165$, $p = .872$). Accordingly, total calling time was higher in HL than in LL rats ($T_{16} = -2.516$, $p = .023$). No difference between groups was observed for the latency to the first call uttered, call duration, peak frequency, peak amplitude, and frequency modulation (all p values $>.100$). Finally, it has to be noted that the difference in call number was not found when nonvocalizing animals were excluded from the analysis ($T_{11} = -1.741$, $p = .110$).

When analyzing call emission per minute, an increase in call number was observed $F(1,10) = 20.375$, $p < .001$; see Figure 10). This increase was accompanied by a group difference, $F(1,16) = 5.334$, $p = .035$), and an interaction between group and time, $F(1,10) = 4.820$, $p < .001$). Subsequent t tests revealed that LL rats vocalized less during min 10 and 11 than HL rats ($T_{16} = -2.324$, $p = .034$ and $T_{16} = -3.264$, $p = .005$; all other p values $>.050$), reflecting a stronger response to repeated shock applications in the latter.

Correlational analyses yielded that the total number of lickings experienced during infancy was significant positively correlated with the time spent calling during min 4–11 ($r = 0.427$, $p = .023$; see Figure 11). A trend for a positive correlation between pup lickings and adult 22-kHz calls was also observed ($r = 0.338$, $p = .078$), showing that rats which had experienced a high level of maternal care during infancy showed more anxiety-related 22-kHz

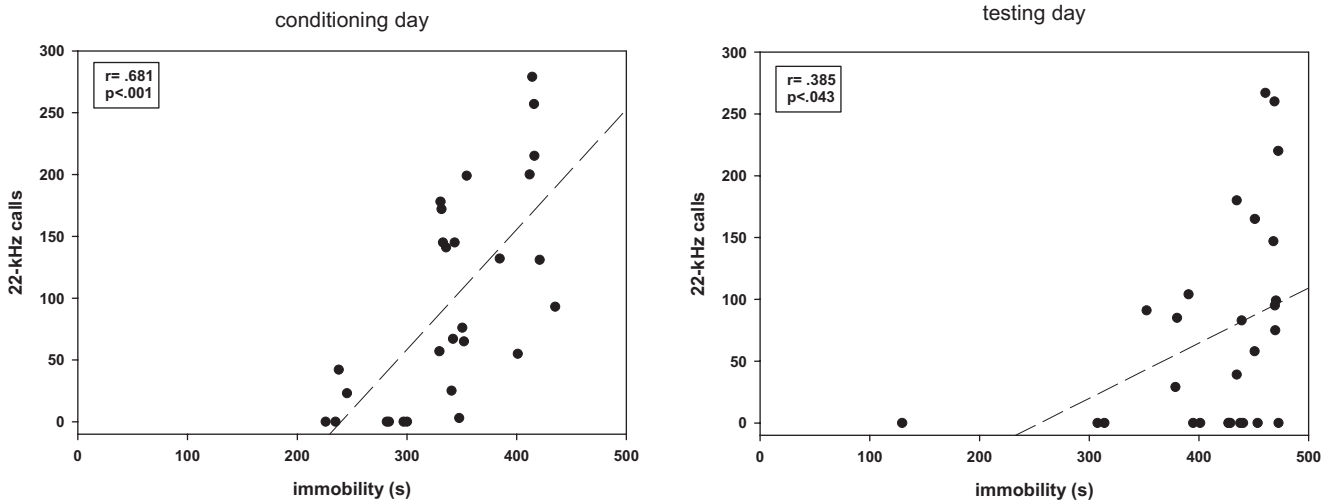

Figure 9. Scatter plots depicting individual relationship between ultrasonic vocalization (22-kHz calls; number) and overt behavior (immobility; in s) displayed in the fear-conditioning paradigm during min 4–11 on the conditioning day (left) and the testing day (right).

Table 6
Fear Conditioning: Behavioral Profile of HL and LL Rats

	Conditioning Day			Testing day		
	HL	LL	*p*	HL	LL	*p*
Immobility (min 1-3 s)	47.55 ± 11.24	32.79 ± 11.04	.368	155.03 ± 11.27	140.23 ± 10.76	.360
Immobility (min 4-11 s)	359.71 ± 16.01	304.09 ± 18.98	.046	441.27 ± 11.95	409.17 ± 15.39	.133

Note. HL = highly licked; LL = low licked. Values reflect means ± *SEM* (HL: $n = 8$, LL: $n = 10$) during conditioning day and testing day.

calls than rats which had been licked less often. In addition, total licking and the number of bouts were positively correlated ($r = 0.384$, $p = .044$; all other p values $>.100$).

On the testing day, the number of animals showing ultrasonic calling was similar (LL: 5 of 10, 50.0%; HL: 5 of 8, 62.5%). However, as on the conditioning day, HL rats showed a more pronounced calling behavior than LL rats. Especially, HL rats started to call earlier than LL rats ($T_8 = 5.456$, $p = .001$), and showed trends for more time vocalizing ($T_{8.750} = -2.014$, $p = .076$), and longer calls ($T_8 = -2.037$, $p = .076$) with higher amplitudes ($T_8 = -2.183$, $p = .061$). In contrast to the conditioning day, however, call number did not differ between groups ($T_{8.811} = -1.617$, $p = .141$). Also no difference between groups was obtained for number of bouts, bout-length, peak frequency, and frequency modulation (all p values $>.100$).

When analyzing call emission over minutes, an increase in call number was observed, $F(1,10) = 11.256$, $p < .001$; see Figure 10. This increase was different between groups, as indicated by a trend for a main effect group, $F(1,16) = 3.103$, $p = .097$, and an interaction between group and time, $F(1,10) = 2.840$, $p = .003$. Subsequent t tests revealed that HL rats vocalized more in the beginning, that is, after the first few tone presentations, than did LL rats (min 7: $T_{16} = -2.313$, $p = .049$; all other p values $>.050$).

Total licking and the latency to utter the first call on the testing day were negatively correlated ($r = -0.679$, $p = .004$). In fact, total licking and total calling time were not only correlated on the conditioning day, but also on the testing day ($r = 0.379$, $p = .047$; see Figure 11; all other p values $>.100$).

Infant ultrasonic calling and behavior during fear conditioning. Infant calling was not correlated with the time rats spent immobile before having experienced tone/shock pairings (call number: $r = 0.088$, $p = .657$, total calling time: $r = 0.060$, $p = .763$). Immobility during shock delivery on the conditioning day (min 4 – 11), however, was negatively associated with infant calling (number: $r = -0.349$, $p = .069$, time: $r = -0.418$, $p = .027$), showing that rats which had vocalized rarely during infancy demonstrated extended immobility to adult tone/shock experiences. Infant calling and immobility on the testing day were not related (all p values $<.100$).

Infant calling and adult calling during fear conditioning were negatively correlated to each other. Thus, infant call number and total calling time were negatively correlated with number ($r = -.429$, $p = .023$ and $r = -.403$, $p = .033$, respectively; see Figure 12) and total time of adult 22-kHz calls ($r = -0.470$, $p = .012$ and $r = -0.435$, $p = .021$, respectively). In contrast, infant calling was not associated with adult calling on the testing day (all p values $<.100$).

Infant overt behavior and behavior during fear conditioning. The only infant behavioral measure which was associated with adult immobility was head-raising (all other p values $>.100$), which was negatively correlated with shock-induced immobility on the conditioning day (min 4–11: $\rho = -.552$, $p = .004$). Such correlations were also observed on the testing day (min 1–3: $\rho = -.475$, $p = .011$ and min 4–11: $\rho = -.566$, $p = .002$).

Apart from a trend for a correlation between the number of infant pivots in isolation and the number of adult 22-kHz calls emitted on the conditioning day ($\rho = -.342$, $p = .075$), head-raising alone was negatively correlated with the number of 22-kHz calls emitted ($\rho = -.458$, $p = .014$) and the time spent calling on the conditioning day ($\rho = -.567$, $p = .002$). It was also negatively correlated with the number of 22-kHz calls emitted ($\rho = -.416$,

Table 7
Fear Conditioning: Ultrasonic Vocalization in HL and LL Rats

	Conditioning day			Testing day		
	HL	LL	*p*	HL	LL	*p*
Latency (s)	320.11 ± 37.71	393.37 ± 21.99	.137	286.05 ± 20.59	463.10 ± 25.08	.001
Calls (*n*)	121.63 ± 31.46	43.10 ± 17.24	.035	89.38 ± 33.57	31.70 ± 12.07	.141
Bouts (*n*)	52.50 ± 11.02	19.20 ± 7.50	.020	41.63 ± 14.16	16.70 ± 5.99	.100
Bout-length (*n*)	1.35 ± 0.47	1.25 ± 0.22	.872	1.34 ± 0.77	0.92 ± 0.38	.641
Total calling time (s)	149.83 ± 34.52	53.03 ± 20.71	.023	129.55 ± 42.19	39.42 ± 14.91	.076
Call duration (s)	1.30 ± 0.07	1.29 ± 0.14	.948	1.60 ± 0.16	1.26 ± 0.05	.076
Peak frequency (kHz)	23.90 ± 0.41	23.44 ± 0.27	.391	23.83 ± 0.58	23.04 ± 0.35	.281
Peak amplitude (HB)	70.88 ± 1.50	67.18 ± 1.81	.141	69.01 ± 1.64	62.53 ± 2.47	.061
Frequency modulation (kHz)	4.42 ± 0.42	3.86 ± 0.66	.475	5.52 ± 1.47	3.93 ± 0.47	.352

Note. HL = highly licked; LL = low licked. Values reflect means ± *SEM* (HL: $n = 8$, LL: $n = 10$) during conditioning day and testing day.

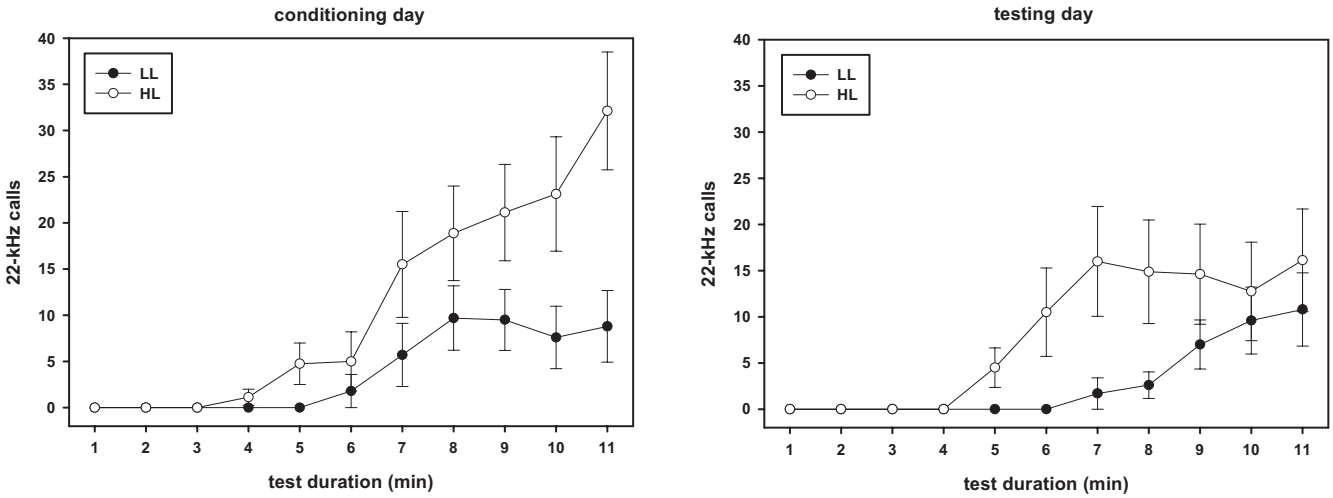

Figure 10. Time courses of ultrasonic vocalization per minute in HL ($n = 8$) and LL rats ($n = 10$). Given are means ± *SEM* for 22-kHz call number on the conditioning day (left) and testing day (right).

$p = .028$) and the time spent calling on the testing day ($\rho = -.449$, $p = .016$).

Discussion

The essential findings of this experiment indicate that there exists a relationship between maternal care, isolation-induced ultrasonic calling by pups and anxiety-related behavior as adults. This was arrived at based on the more specific results obtained, which showed that: Playback of isolation-induced calling efficiently induced maternal search behavior (1). Individual differences in infant isolation-induced calling were strongly linked to maternal care (2), and to infant overt behavior in isolation (3). Infant overt behavior itself, however, was only weakly associated with maternal care (4). Furthermore, adult anxiety-related behavior was negatively correlated with infant isolation-induced calling (5), and maternal care (6).

Important to note, influences of handling or marking during the first days of life were not evident. Comparisons between experimental and control pups showed that differences in maternal licking behavior, pup's overt and calling behavior in isolation, and adult overt behavior in the activity box, the elevated plus maze, and during fear conditioning were virtually absent. Also adult ultrasonic calling during fear conditioning did not differ between groups, except that control animals showed lower call frequency when compared to experimental animals on the testing day. Further, the position of markings did not affect maternal licking, which is in line with the virtual absence of differences between

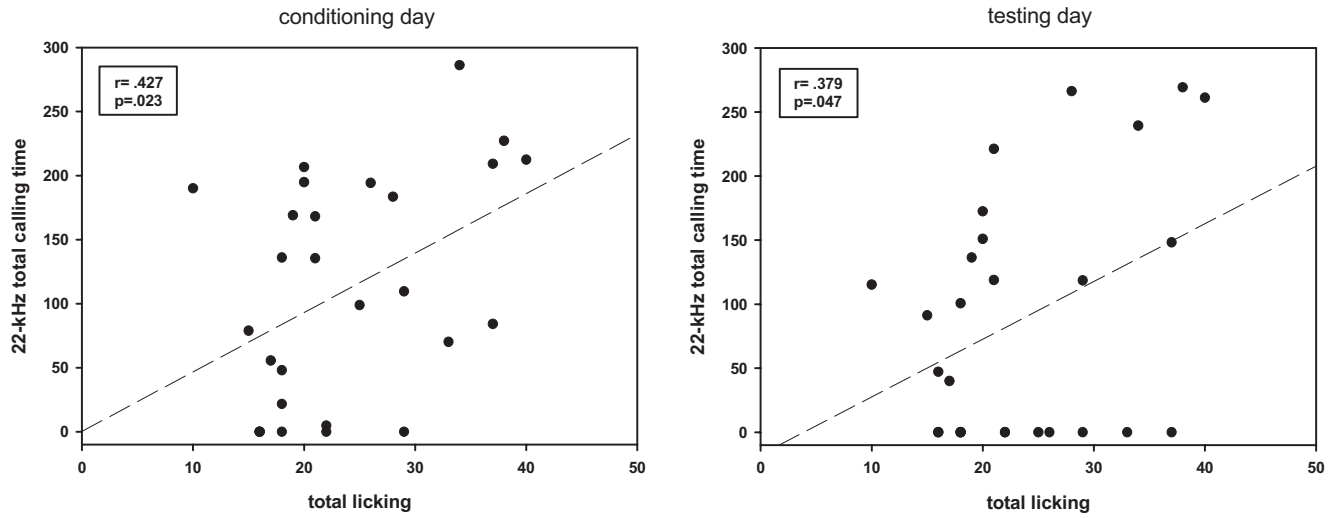

Figure 11. Scatter plots depicting individual relationship between ultrasonic vocalization (22-kHz total calling time; in s) displayed in the fear conditioning paradigm during min 4–11 on the conditioning day (left) and the testing day (right) and maternal care (total licking; number of anogenital and body licking during the total observational period, i.e., 24 h).

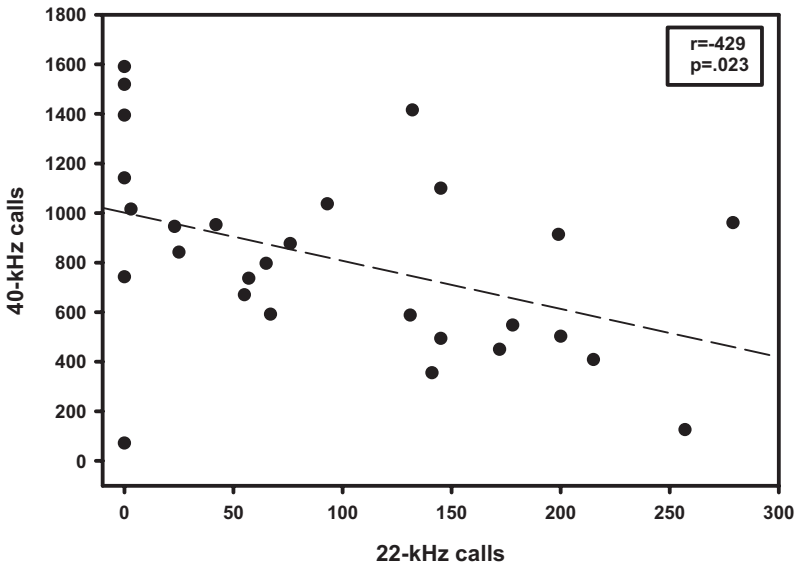

Figure 12. Scatter plot depicting individual relationship between ultrasonic vocalization (40-kHz calls; number) displayed during the 10 min of isolation in infancy and ultrasonic vocalization (22-kHz calls; number) displayed in the fear conditioning paradigm in adulthood during min 4–11 on the conditioning day.

control and experimental pups. Therefore, the present results can be generalized to nonmanipulated male rats. Even the use of all-male litters does not restrict validity, since mothers interact similarly with males, irrespective of whether they are housed together with females or not (Moore & Morelli, 1979). Finally, although it is known that maternal behavior can be influenced by litter size (Seitz, 1954), results on effects of litter size on emotionality are quite inconsistent. In some studies rats of small litters were less emotional (Hinz, Hecht, Rohde, & Dörner, 1983; Seitz, 1954), in some other studies they were more emotional (Dimitsantos et al., 2007; Robinson, 1975), and other studies did not observe behavioral differences (Broadhurst & Levine, 1963; Grota & Ader, 1969b).

Playback of Isolation-Induced Calling Induces Maternal Search Behavior

It is known for several years that infant rodents emit ultrasonic calls when isolated from mother and nest (Noirot, 1968; Zippelius & Schleidt, 1956), and already then it was postulated that isolation-induced calling can induce retrieval behavior (Zippelius & Schleidt, 1956). The present findings support this hypothesis, since playback of 40-kHz calls induced stimulus-directed locomotor activity. Importantly, this activation was stimulus-dependent, since arms in front of the stimulus source were preferred only during playback of 40-kHz calls.

The present findings are in contrast to observations by Smotherman et al. (1974), who found that lactating females do not prefer the arm of a Y maze where 40-kHz calls were presented when compared to the arm without tone presentation. Since they observed stimulus-directed search behavior only when ultrasonic calls were accompanied by relevant olfactory cues, they postulated that olfactory cues from a displaced pup are "a necessary condition for retrieval" (Smotherman et al., 1974, p. 61). Recently, this assumption was supported by Farrell and Alberts (2002), who found that lactating females do not spent more time in front of an ultrasonic speaker when 40-kHz calls were presented in comparison to the time where no calls were presented. However, the present findings clearly show that search behavior can occur even without the presence of olfactory cues, and this finding is in accordance with a wealth of evidence in mice (Ehret & Haack, 1981; Sewell, 1970; for a more detailed discussion see also: Hahn & Lavooy, 2005). Furthermore, the present findings show that ultrasonic vocalizations can serve as stimuli for pup localization, which is in accordance to findings by Allin and Banks (1972) and Brunelli et al. (1994), who found that rat mothers mainly searched for their pups in those areas where 40-kHz calls were presented. In addition, the present finding that 40-kHz sine wave tones were inefficient to induce search behavior supports the hypothesis of Brudzynksi et al. (1999) that specific call features, like the extent of frequency modulation, are critical to induce retrieval behavior, since alternating frequency sweeps can be more easily detected than a steady sound.

Individual Differences in Isolation-Induced Calling are Linked to Maternal Care

Huge individual differences in isolation-induced infant calling have repeatedly been observed before (Brunelli & Hofer, 1996; Graham & Letz, 1979; Hofer & Shair, 1978; Myers et al., 2004). Part of this variability could be due to genetic factors, since rats can be bred successfully for high or low rates of calling in isolation (Brunelli, 2005; Brunelli, Myers, Asekoff, Hofer, 2002, Brunelli, Hofer, & Weller, 2001; Brunelli et al., 1997; Hofer, Shair, Masmela, & Brunelli, 2001). However, even within these lines, high levels of variability have been found, for example, Brunelli et al. (1997) observed that call rates ranged between 0 and 700 per min in the line selected for high rates of calling. Thus, it seems likely

that epigenetic factors hold strong influence on isolation-induced calling, and the present results indicate that maternal care may be one such factor.

The present results show that rat pups raised by mothers, that demonstrated pronounced approach behavior in response to playback of 40-kHz calls, called less in isolation than pups raised by mothers with weak or no approach behavior. Interestingly, the association between maternal responsiveness and infant calling behavior in isolation was only observed when acoustic stimuli were presented alone, but not in the retrieval test where acoustic and olfactory stimuli were present. This finding shows that not the maternal responsiveness per se, but the maternal responsiveness toward 40-kHz calls is related to calling in isolation. Thus, it can be important to separate different factors occurring in the natural environment of a mother, to understand the relationship between maternal care and infant behavior. The finding that maternal responsiveness is negatively related to isolation-induced calling is corroborated by findings of D'Amato, Scalera, Sarli, and Moles (2005) who showed that pups of mice mothers that scored higher in maternal responsiveness emit lower call rates than pups of mothers with a comparatively low maternal responsiveness.

Furthermore, it was found that maternal licking is strongly linked to isolation-induced infant calling. Thus, HL pups emitted less calls in isolation than LL pups. A detailed analysis of ultrasonic calls revealed that apart from call number and total calling time, several call characteristics differed between both subgroups, since HL pups emitted fewer and shorter bouts with calls being characterized by lower amplitudes and shorter durations. However, they emitted calls which were more frequency modulated. The finding that maternal care is related to isolation-induced calling seems to contrast results of cross-fostering studies (Brunelli et al., 2001; Graham & Letz, 1979). Brunelli et al. (2001) showed that postnatal maternal effects do not modulate call rates in animals selected for high or low calling rates. However, it has to be noted that a within-litter selection procedure was used for breeding of these animals to minimize maternal effects (Hofer et al., 2001). In contrast, Rojowski, Weller, Hofer, & Brunelli (2000) found that high line dams showed a reduced maternal responsiveness toward pups than random or low line dams, which is in accordance to the present findings. Furthermore, the negative relationship between maternal care and ultrasonic calling found here also seems to go against observations by Broutte-Lahlou et al. (1992), who showed that playback of ultrasonic calls can evoke licking behavior in the rat. These conflicting results could be due to the fact that all acoustic signals above 10 kHz were considered as ultrasonic calls by these authors, who also did not verify call presentation, leading to the assumption that licking behavior was induced by calls other than 40-kHz ones. On the other hand, both findings may not be in conflict, when considering that here HL pups call less, but more efficiently, since their calls were more frequency modulated. In fact, Brudzynski et al. (1999) have argued that call characteristics, such as extent of frequency modulation are critical to induce maternal care. Furthermore, a negative relationship between maternal care and isolation-induced calling, as found here, has been reported by Darnaudery et al. (2004), who studied the effects of cross-fostering on maternal care and infant behavior and found that fostering dams showed more maternal care than real mothers. Interestingly, pups raised by fostering dams showed less isolation-induced calling when compared to pups raised by real mothers.

In all, the present findings indicate that maternal responsiveness and maternal care are factors that can tune calling behavior in offspring. Importantly, the relation between high levels of maternal care and low calling in isolation seems to be anxiolytic. Anxiolytics, especially benzodiazepines, are known to reduce the number of calls emitted in isolation (Gardner, 1985). Furthermore, Insel et al. (1986) have shown that diazepam not only reduces call number, but also call amplitude, while pentylentetrazol, an anxiogenic substance, induced an increase in call number and call amplitude. In fact, several studies have shown that maternal care can have profound influences on anxiety-related behavior. Thus, rats licked more often by mothers, showed decreased startle responses (Zhang et al., 2005), increased open field exploration (Caldji et al., 1998; Francis et al., 1999), and shorter latencies to eat food provided in a novel environment (Caldji et al., 1998) than did pups that were licked less often. Moreover, the former showed less burying in a defensive burying paradigm, fewer defensive responses in a resident-intruder test, and less shock-induced freezing in comparison to rarely licked rats (Menard et al., 2004, 2007). Interestingly, these behavioral differences are accompanied by alternations in stress reactivity (Liu et al., 1997) and various neural changes in brain areas implicated in anxiety regulation (Liu et al., 1997; Caldji et al., 1998). Thus, the present finding that maternal licking is negatively linked to isolation-induced calling is in accordance with a wealth of evidence that maternal licking is negatively associated with anxiety-related behavior in adulthood. The fact that differences in individual dispositions in anxiety-related behavior using ultrasonic vocalization are already detectable in infant rats, is in line with the finding that physiological changes are also already detectable in infancy, namely as soon as pnd 6 (Fish, Diorio, Champagne, & Meaney, 2005).

Individual Differences in Isolation-Induced Calling are Linked to Overt Behavior in Isolation

In accordance to Hofer and Shair (1978) pups showed sustained levels of activity throughout the period of isolation. Interestingly, Hofer and Shair (1978) found that call rates were higher in time-periods where animals showed locomotion in comparison to time-periods without locomotor activity, suggesting a positive relationship between calling and overt behavior. However, when analyzing individual levels of calling and overt behavior, only a weak association was found (Brunelli, 2005; Brunelli & Hofer, 1996).

In the present study, locomotor activity was highly correlated with ultrasonic calling, especially in case of pivoting. Hofer (1996, p. 205) suggested that such pivoting of isolated pups "provides a means of projecting the ultrasonic "beam" over a wide directional range". Apart from pivoting, the number of squares crossed and head raising were also positively correlated with calling behavior, whereas twitching and grooming were not. Thus, in accordance to the assumption by Hofer (1996), only those types of behavioral activity, which are helpful for call transmission are positively related to call production. This is in line with a slow motion analysis of neonatal behaviors associated with calling in mice pups, where Branchi, Santucci, Puopolo, and Alleva (2003) found an increase of head-raisings immediately prior to call emission.

Infant Overt Behavior Is Only Weakly Associated With Maternal Care

Despite the fact that overt activity and calling behavior were highly correlated, maternal care was only weakly linked to overt behavior. LL pups showed a similar number of twitches, rears, head-raisings, pivots, and square crosses as HL pups. The latter, however, spent more time grooming than the former. However, little is known about the contextual control of grooming in infancy (for a discussion on grooming in adulthood, see Brunelli & Hofer, 1996).

Adult Anxiety-related Behavior Is Negatively Correlated With Infant Isolation-Induced Calling

Based on the above findings, it could be assumed that isolation-induced infant calling is a predictor of anxiety-related behavior in adulthood. Actually, the comparison between rats bred for high or low rates of isolation-induced calling indicates such a relationship. It was found that the former resemble a more anxious phenotype with higher rates of defecation and urination (Brunelli et al., 1997), enhanced latencies to enter a new arena (Zimmerberg et al., 2005), less struggling but more immobility in a Porsolt forced swim test (Zimmerberg et al., 2005), and more ultrasonic calls in response to handling and touch (Brunelli, 2005). Furthermore, animals bred for an anxious phenotype show more isolation-induced vocalizations than animals bred for a less anxious phenotype (Insel & Hill, 1987; Naito, Inoue, & Makino, 2000; Wigger, Loerscher, Weissenbacher, Holsboer, & Landgraf, 2001).

In the present study, infant ultrasonic calling was only loosely associated to overt behavior in the activity box and was not related to plus maze behavior. This is in contrast to findings by Dichter, Brunelli, and Hofer (1996) who found that animals bred for low or high rates of isolation-induced calling differ in their anxiety-related behavior on the elevated plus maze. However, it has to be mentioned that high and low line pups used in the study by Dichter et al. (1996) did not differ in infant calling behavior and further replications failed (Rojowski, Brunelli, Shair, & Hofer, 1999; Shair, Brunelli, Velazquez, & Hofer, 2000). One study even found a negative relationship between infant calling and anxiety-related behavior in the elevated plus maze (Shair et al., 2000). Thus, Brunelli (2005, p. 62) summarized "the high line has shown erratic performances in the elevated plus maze, inconsistent with an "anxiety" phenotype."

Here, strong evidence for a negative relationship between infant isolation-induced calling and adult anxiety-related behavior was found, given the negative correlation between infant calling and immobility during fear conditioning, indicating that rats which had rarely vocalized during infancy showed enhanced immobility to adult tone/shock experiences. Furthermore, infant calling was negatively correlated with adult calling during fear conditioning, implicating that rats, which had vocalized rarely during infancy emitted numerous 22-kHz calls in response to tone/shock experiences in adulthood. Recent studies have shown that overt and calling behavior in the fear conditioning paradigm depend on external and internal factors, namely shock intensity (Wöhr et al., 2005) and individual disposition of anxiety-related behavior as measured on the elevated plus maze (Borta et al., 2006). Interestingly, it was also found that this disposition is negatively related to isolation-induced calling (Schwarting & Pawlak, 2004), which is in accordance with the present finding of more adult anxiety-related behavior in animals with low call rates in infancy. The discrepancy to the findings in animals bred for high versus low lines of infant calling, or high versus low anxiety-related behavior in adulthood might be explained by the fact that the normal variation in unselected animals was used in the present study and not the extremes of several generations of breeding. Another explanation could be that the emission of ultrasonic calls in infancy is part of an active coping style as shown in high positive correlations between calling and overt behavior, whereas in adulthood, call emission is part of a passive coping style as shown in high positive correlations between calling and immobility in the present study as in a previous one (Wöhr et al., 2005; for a discussion of coping styles in the context of ultrasonic calling, see Brunelli, 2005). In line with this assumption, it was also shown here that isolation-induced activity is negatively related to adult immobility during fear conditioning, meaning that animals that responded with locomotor activation toward aversive stimuli in infancy showed the same pattern in adulthood. Remarkably, such an association was not found between infant overt behavior and adult overt behavior during testing for activity, indicating that the behavioral effects observed during isolation and fear conditioning are part of a stress response, whereas the behavioral activation observed during testing in novel, but unthreatening situations reflects the need for exploration. In fact, a number of factor analyses have revealed the existence of distinct dimensions for behavior shown in novel, but unthreatening situations, namely emotionality and exploration, that is, activity (e.g., Whimbey & Denenberg, 1967; for review, see Ramos & Mormède, 1998); a finding which is supported by the absence of positive correlations between activity in novel situations and anxiety-related behavior in various tests (Ho, Eichendorff, & Schwarting, 2002).

Adult Anxiety-related Behavior Is Negatively Correlated With Maternal Care

It is known that maternal care can have pronounced effects on adult anxiety-related behavior (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005). In the present study, however, no effect of maternal care on activity box and plus maze behavior was found. The latter is surprising, since the elevated plus maze is a widely accepted test for anxiety, which is characterized by bidirectional drug sensitivity (Carobrez & Bertoglio, 2005). Also, the test is useful to detect individual dispositions of anxiety-related behavior (Schwarting & Pawlak, 2004) which are related to differences in other behavioral tests, where anxiety and aversion play a critical role, like object burying (Ho et al., 2002), active avoidance (Ho et al., 2002; Ho, Pawlak, Guo, & Schwarting, 2004), and fear conditioning (Borta et al., 2006).

Conversely, an effect of maternal care was observed during fear conditioning, but in the opposite direction of that described in the literature (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005), since LL, but not HL rats, showed a reduced anxiety-related behavior. Before the first shock was given, rats of both subgroups did not differ, but with beginning of shock delivery, HL rats showed more pronounced calling behavior, particularly HL rats emitted more than twice as many 22-kHz calls than LL rats. However, the group difference in calling time is

primarily due to call likelihood, but not quantity (see also Borta et al., 2006). Interestingly, the lower likelihood to call during shock delivery in rarely licked animals was accompanied by a lower level of immobility. In accordance with the differences between the subgroups, correlational analyses yielded that the total number of lickings experienced as pups was positively correlated with the emission of 22-kHz calls and the time spent immobile. On the testing day, however, group differences were weaker. Thus, apart from a shorter latency to start calling in HL, reduced calling behavior in HL rats was solely indicated by trends for shorter total calling time and shorter call durations; a result, which was confirmed by a correlational analysis. Importantly, however, total licking and total calling time were again positively correlated. The paucity of differences between both subgroups in ultrasonic calling on testing day is paralleled by a lack of differences in the behavioral profile; there were also no substantial correlations between total licking and immobility.

The fact that HL rats showed more pronounced anxiety-related behavior than LL rats goes against several studies which show that animals that experienced more maternal licking display lower levels of anxiety-related behaviors as adults (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005). The discrepancy between the present and previous research may be due to the experimental task used. In fact, studies using fear conditioning obtained comparable weak effects of maternal care on fear learning (Bagot & Meaney, 2005). Animals, which were licked rarely during infancy, displayed more immobility when returned to a context in which a single shock had been previously presented than animals which were licked often, but equivalent levels of immobility were observed to a tone previously paired with a single shock or multiple shocks, as well as in a context in which multiple shocks had been presented. Therefore, it might be that attenuating effects of maternal care on emotionality are predominantly evident in unconditioned tests of anxiety, whereas maternal care may have no or opposite effects in conditioned tests. Emotionality in fact has been shown to be a multidimensional concept (for review, see Ramos & Mormède, 1998), and it is important to differentiate between fear and anxiety (e.g., Waddell, Morris, & Bouton, 2006; for review, see Walker, Toufexis, & Davis, 2003) as between unconditioned and conditioned tests (e.g., Baratta et al., 2007; for review, see Rosen & Donley, 2006). One obvious difference between unconditioned and conditioned tests is the involvement of cognitive processes. Thus, it might be that anxiety-related behavior in unconditioned tests is primarily dependent on the individual level of emotionality, whereas in conditioned tests anxiety-related behavior is dependent on both, the individual level of emotionality and cognitive abilities. The fact that highly licked animals have a reduced level of emotionality (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005), but a superior level of cognitive abilities (Liu, Dorio, Day, Francis, & Meaney, 2000; Bredy, Grant, Champagne, & Meaney, 2003a, Bredy, Humpartzoomian, Cain, & Meaney, 2003b; Bredy, Zhang, Grant, Diorio, & Meaney, 2004), might result in an equal level of anxiety-related behavior as in rarely licked animals where an enhanced emotionality is accompanied by lower cognitive abilities. In line with this explanation it was found that highly licked animals showed a faster extinction on the test day than rarely licked animals, indicating in fact superior cognitive abilities in the former.

Besides this, the present disparate findings may be related to a host of factors that include methodological differences and strain. Wistar rats were used in the present experiment, whereas previous research has been conducted on Long-Evans hooded rats (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005), and it is known that maternal care displayed by Wistar rats differs from that of Long-Evans rats (McIver & Jeffrey, 1967). However, methodological differences are more likely to be the reason for incongruities. In contrast to previous research (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005), pups were ranked according to the individual number of maternal lickings received, instead of the overall licking behavior of the mother. Thus, it is possible that within-litter variability in the amount of dam licking received by a pup reflects individual differences in pups to which the mother is responding. Indeed, it is likely that infant behavior can influence maternal licking (Moore & Chadwick-Dias, 1986), and anogenital licking was designated as the major source of individual variation in stimulation that is received within the same litter (Moore & Power, 1992). Therefore, maternal licking as measured in the present study might be partly different from maternal licking in other studies, where pups were assigned to groups according to the mother (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005). Moreover, offspring was designated as HL or LL if the number of lickings experienced was below the first quartile or above the third quartile, whereas previous research used a cut-off of one *SD* (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004, 2007; Zhang et al., 2005). Litter effects may also be another reason for the present counterintuitive findings (Holson & Pearce, 1992; Zorrilla, 1997). Monte-Carlo simulations have shown that under conditions that are typical in developmental studies, that is, 4 dams per group with 2 pups per dam and an alpha-level of 5%, such litter effects can result in a false positive rate of about 15–20% (Holson & Pearce, 1992). Although such typical conditions are given in the present study, since each of the HL and LL subgroups contained 4 mothers with about 2 pups per mother on average, it is unlikely that present findings are based on a litter effect. First, because the amount of significant effects when comparing HL and LL subgroups (about 27%) clearly exceeds what can be expected on basis of litter effects. Second, the picture of the present findings is not likely to be explained by litter effects, since differences between control and experimental animals were virtually absent; instead, as expected, differences were solely detected when HL and LL subgroups were compared. Finally, the occurrence of huge within-litter variability shows that litter effects cannot solely be responsible for the present findings.

The importance of factors, like group assignment, strain, and the experimental task used, is indicated by counterintuitive results in a series of studies. In accordance with present findings, there are other studies showing that high levels of maternal care can result in a more anxious phenotype in adulthood. For instance, Clinton et al. (2007) have observed a more pronounced behavioral inhibition and more anxiety-related behavior in adult Sprague–Dawley rats which were raised by highly attentive mothers in comparison to less attentive ones. Remarkably, the former constantly spent a more time licking, grooming, arched-back nursing, and nesting throughout the first two weeks after birth. A similar picture was obtained by Dimitsantos et al. (2007). They observed that Sprague–Dawley rats, that grew up in small litters and therefore

experienced more maternal care than rats which grew up in huge litters, showed more anxiety-related behavior in adulthood when compared to the latter. Moreover, Birke and Sadler (1987) showed in Wistar rats that an experimentally induced reduction of licking by usage of perfume did not result in more anxiety-related behavior, but instead in animals which showed much higher levels of social play than controls (see also Moore & Power, 1992; Olesen, Bychowski, Auger, & Auger, 2007). These behavioral studies are complemented by a study by Barha, Pawluski, and Galea (2007) who showed that endocrine stress reactivity can also be more pronounced in Sprague–Dawley rats raised by attentive mothers when compared to less attentive mothers.

Conclusions

Consistent with reports of individual differences in adult anxiety-related behavior, offspring of mothers with relatively low levels of maternal care and maternal responsiveness toward isolation-induced infant vocalizations appeared to be more anxious, since they emitted more calls when separated from their mother and litter than pups of mothers with high levels of maternal care and maternal responsiveness. Due to the fact that maternal care had comparable weak effects on overt behavior, measuring such ultrasonic vocalizations can provide information about an affective trait of the rat, which might be difficult to obtain by overt behavioral parameters. This provides an opportunity to study the development of emotionality from early life onward.

Moreover, this study shows that maternal care and infant ultrasonic calling is negatively correlated with adult overt behavior and ultrasonic calling in aversive situations. This counterintuitive result shows that it might be of great importance to dissect the concept of emotionality and to include coping styles into the interpretation. In total, the present results indicate that low levels of maternal care promotes an active coping style, characterized by strong behavioral activation and calling in infancy when separated from mother and litter, and less behavioral inhibition during fear conditioning in adulthood.

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Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: An embryo-transfer study

Running head: Embryo-transfer study on ultrasonic calling in mouse pups

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Abstract

Infant rodents emit ultrasonic vocalizations when isolated from dam and littermates. Due to the context of their occurrence and the well described bidirectional modulation by substances known for their capability to influence emotionality, it was postulated that such calls reflect a negative affective state akin anxiety. Comparative studies observed pronounced differences in calling behavior between strains, which were paralleled by differences in maternal care. Therefore, it was recently hypothesized that early environmental factors may have strong impact on call production. Here, the relative contributions of genetic background, gender, and early environmental factors on calling behavior in C57BL/6JOlaHsd and C57BL/6NCrl were studied by using an embryo-transfer procedure. The results show that these sub-strains differ in the amount of calling and specific call features, like call frequency and amplitude. The embryo-transfer procedure indicated that the observed differences in the amount of ultrasonic calling are strongly dependent on the dyadic interaction between mother and pup. Conversely, call features were primarily dependent on the genotype of the pup. Thus, call frequency and frequency modulation were solely dependent on the pup, i.e. its genotype and gender. However, there was one exception, namely call amplitude, which was solely dependent on the genotype of the mother. In total, it can be concluded that both genomic and nongenomic factors can tune calling behavior in mouse pups.

Key words: ultrasonic vocalization (USV), maternal care, pup retrieval, individuality, anxiety, alpha-synuclein, embryo-transfer, epigenetic effects, sex, strain differences, inbred, C57BL/6N, C57BL/6JOla

1. Introduction

Infant rodents emit ultrasonic vocalizations when isolated from dam and littermates (e.g. Zippelius & Schleidt, 1956; for review see: Constantini et al., 2006). Such calls play an important role in pup survival, since they can elicit maternal behavior, like retrieval (Allin & Banks, 1972; Sewell, 1970; Smith, 1976; Smotherman et al., 1974; Wöhr & Schwarting, in press; for review see: Ehret, 2005). Importantly, isolation-induced ultrasonic vocalizations seem to reflect a negative affective state akin anxiety, since they are modulated by anxiogenic and anxiolytic drugs (Gardner, 1985; Insel et al., 1986; for review see: Hofer, 1996). Also, these pup vocalizations have been proposed as sensitive markers to evaluate alterations of neurobehavioral development (Branchi et al., 2001). Therefore, they have received increasing experimental attention, for example, to examine the respective roles of genetic, maternal and other environmental influences.

The importance of genetic effects was indicated by early studies where differences between species and strains were observed (Sales & Smith, 1978). Within the species Mus musculus, inbred strain differences in call rate and call characteristics have been consistently observed (Bell et al., 1972; Cohen-Salmon et al., 1985; Hennessy et al., 1980; Robinson & D'Udine, 1982), and genetic studies have shown, in summary, that call rate and probably all acoustic call characteristics have a multiple genetic background (Hahn et al., 1987; Hahn et al., 1997; Hahn et al., 1998; Hahn & Schanz, 2002; Roubertoux et al., 1996; Thornton et al., 2005). Ehret (2005) explained this observation by the fact that genes in three main areas of the infant development may affect ultrasonic vocalizations, namely genes, which contribute to the perceptual pathways of the nervous system that are responsible for the perception of the releasing stimuli, genes that are involved in the regulation of emotion and motivation, and genes that are linked to the anatomical properties of the breathing system and larynx. The multitude of genetic influences on sound production was also observed in studies on knockout mice. There it was found that mice with demyelization (Bolivar and Brown, 1994), mice

lacking *Foxp2* (Shu et al., 2005), *MeCP2* (Picker et al., 2006), oxytocin (Winslow et al., 2000), or different receptors, like mu-opioid (Moles et al., 2004), vasopressin 1b (Scattoni et al., 2007), 5-HT_{1A} (Weller et al., 2003), 5-HT_{1B} (Brunner et al., 1999; El-Khodor et al., 2004; Weller et al., 2003), and CB_1 (Fride et al., 2005) show altered calling behavior in infancy.

Besides, numerous environmental variables, in particular maternal care, have also been shown to modulate ultrasonic calling in rodents. Hofer and Shair (1978; 1980; for review see: Hofer, 1996) showed that the mere presence of the dam acutely inhibits ultrasonic calling. Moreover, brief interactions of the pup with its dam can induce an intensified vocal response during subsequent isolation (Hofer et al., 1994; Hofer et al., 1999; Moles et al., 2004; Myers et al. 2004; Shair et al., 1997; Shair et al., 2003; for review see: Shair, 2007). Apart from acute and short-term effects, however, there are also data suggesting that maternal behavior can have long-term effects on ultrasonic calling of pups during isolation. Such long-term effects were indicated by genetic analyses, where small but persistent maternal effects on call rate, duration, frequency, and frequency modulation were observed (Roubertoux et al., 1996; Thornton et al., 2005). A possible mechanism for maternal effects on ultrasonic calling was observed by D'Amato and Populin (1987) who found that call rate of normal mouse pups was reduced when reared by deaf mothers, indicating that the absence of an adequate response by the mothers can result in a reduction of calling behavior. However, in pups raised by normal mothers, reduced calling rates may not result from the absence of adequate maternal responses, but instead from a sustained level of maternal care yielding anxiolytic-like effects. Recently, D'Amato et al. (2005) demonstrated that pups raised by mothers from the more responsive C57BL/6 strain elicited fewer isolation-induced calls than those raised by the less responsive BALB/c strain.

Strain differences in mice have been reported for several measures of maternal behavior, like pup retrieval, nest building, nursing, and licking (Carlier et al., 1982; Cohen-Salmon et al., 1985; Champagne et al., 2007; Hennessy et al., 1980). Evidence for maternal

effects on offspring development came from reciprocal breeding of inbred mouse strains (Calatayud et al., 2001; Calatayud et al., 2004) and cross-fostering studies (Francis et al., 2003; Priebe et al., 2005; Zaharia et al., 1996; for review see: Gordon & Hen, 2004). By using an embryo-transfer, Francis et al. (2003) were able to show that epigenetic factors hold strong influence on anxiety-related behavior in adult mice. From rat studies it is known that variations in the nursing style affect the development of stable individual differences in emotionality (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004; Menard et al., 2007; Zhang et al., 2005), and that isolation-induced calling is a sensitive marker for differences in maternal licking experienced throughout the first week of life (Wöhr & Schwarting, in press).

The objective of the present study was to assess potential causes of individual differences in various characteristics of pup ultrasonic vocalizations in C57BL/6 mice. To dissociate between effects of genetic background and maternal care, an embryo-transfer was conducted, where blastocysts of C57BL/6JOlaHsd (B6JOla) and C57BL/6NCrl (B6N) were transferred to pseudo-pregnant females either of the same or the other sub-strain. These sub-strains were selected since it is known that they differ in adult anxiety-related behavior, namely the course of extinction of conditioned fear. Thus, C57BL/6JOla develop lower levels of freezing to the context where they have been shocked before, and their maximal fear responses were restricted to a shorter period of time (Radulovic et al., 1998; Siegmund et al., 2005; Siegmund & Wotjak, 2007; Stiedl et al., 1999), reflecting a different susceptibility to develop symptoms resembling those in posttraumatic stress disorder (PTSD; Siegmund and Wotjak, 2007).

2. Materials and methods

2.1. Animals and housing: C57BL/6NCrl (B6N) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and C57BL/6JOlaHsd (B6JOla) mice were purchased from Harlan-Winkelmann (Borchen, Germany). All mice were housed in type 2 long Macrolon cages in the specified pathogen free mouse facility of the Gene Centre in Munich. Water and food (Ssniff, Germany) were freely available. Room temperature was 25°C with 40% humidity and a 12-h light/12-h dark cycle (lights on at 7 AM). All experiments and experimental procedures were approved by the Committee on Animal Health and Care of the local governmental body of the state of Bavaria (Regierung von Oberbayern) and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals.

2.2. General methods: By using an embryo-transfer, four developmental conditions were created (Donor strain > Recipient strain): B6JOla > B6JOla (n: males = 17, females = 16; 6 litters), B6JOla > B6N (n: males = 7, females = 9; 4 litters), B6N > B6N (n: males = 27, females = 18; 7 litters), B6N > B6JOla (n: males = 12, females = 8; 3 litters). Pregnant females were monitored for birth. Within 12 hours of birth [postnatal day (pnd) 0] litters with more than 10 pups were reduced to 10 animals per litter by discarding surpass pups. Thereafter, animals remained undisturbed until behavioral tests started. On pnd 7, pups were screened for isolation-induced ultrasonic calling and maternal retrieval behavior was measured. Behavioral tests were conducted between 8 am and 7 pm in a separate room.

2.3. Embryo-transfer: For the production of the embryos, 8-week old females were mated with males of the same mouse sub-strain. The females were screened for vaginal plugs every morning and evening. Females were killed at day 3 after finding a vaginal plug (3.5 dpc) through cervical dislocation. The uterus was removed and flushed with M2 medium containing 0.4% bovine serum albumin (BSA) and the blastocysts were collected under a

stereomicroscope with 20x magnification (Nagy et al., 2003). The embryos were transferred to M2 medium with 0.4% BSA microdrops on a culture dish covered with paraffin oil at 37°C until needed. Between 12 and 20 embryos were transferred into the uterus of a pseudo-pregnant female recipient (2.5 dpc) which was prepared by mating 12-week-old females with vasectomized males. The skin and muscles of the anesthetized recipient were cut and the uterus externalized from the peritoneal cavity. Under a stereomicroscope with 20x magnification, the uterus was punched with a needle near the oviduct. A transfer pipette prepared with M2 medium and the embryos was inserted through the punched whole and the embryos were placed into the uterus. Embryos of one mouse sub-strain were transferred to recipients of the same mouse sub-strain and to recipients of the other sub-strain, depending on the experimental group.

2.4. Maternal retrieval behavior: To induce maternal retrieval behavior, all pups of a given litter were removed from the nest and placed in the edge most distal from the nest on pnd 7. The latency to pick up the first pup and the latency to retrieve the first and last pup were measured.

2.5. Isolation: To induce ultrasonic vocalization, pups were isolated for 5 min from the mother and nest on pnd 7. Pups were individually removed from the nest in random order and gently placed into a dish (8 x 8 x 3 cm) on a warming plate at 27°C. The dish was placed in a sound attenuating chamber (55 x 65 x 50 cm), which was prepared with sound absorbent foam inside and covered outside with aluminum foil. Ultrasonic vocalization was recorded using an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Germany) suspended 7 cm from the testing surface. The microphone was sensitive to frequencies of 15-180 kHz with a flat frequency response (± 6 dB) between 25-140 kHz. It was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, where

acoustic data were displayed in real time by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics), and were recorded with a sampling rate of 300,000 Hz in16 bit format.

After recording, the pups were marked for identification by foot tattoo with black drawing ink (Pelikan, Germany). The dish was cleaned with Bacillol AF after each session. After replacing the pup into the cage 5 min were allowed to elapse until going on with the next littermate.

2.6. Analysis of ultrasonic vocalization: For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transformation was conducted (512 FFT-length, 100 % frame, Hamming window and 75 % time window overlap). Spectrograms were produced at 586 Hz of frequency resolution and 0.427 ms of time resolution.

Call detection was provided by an automatic threshold-based algorithm (threshold: -40 dB) and a hold-time mechanism (hold time: 10 ms). Since no ultrasonic vocalizations were detected below 30 kHz, a lower-cut-off-frequency of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of call detection was verified by an experienced user. When necessary, missed calls were marked by hand to be included in the automatic parameter analysis. Various parameters, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element, were determined automatically. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. The extent of frequency modulation, i.e. the difference between the lowest and the highest peak frequency within each call was also measured automatically. Temporal parameters determined included call duration, total calling time, and the duration of intervals between subsequent calls. Finally, the total number of calls emitted was measured.

2.7. Statistical analysis: To determine call duration, peak frequency, peak amplitude, and the extent of frequency modulation, the mean of each call parameter served as the statistical unit in each subject. To test whether B6JOla and B6N pups differ in their calling behavior following within-strain transfer, a two-way analysis of variance (ANOVA) with the factors sub-strain of the pup and gender was used. Maternal retrieval behavior was compared between B6JOla and B6N pups by using an independent t-test. To determine the contribution of early environmental factors and genetic predispositions to ultrasonic calling behavior in these two sub-strains, a three-way ANOVA with the factors sub-strain of the pup, i.e. genotype, sub-strain of the mother, and gender was performed. Maternal retrieval behavior was compared between B6JOla and B6N pups in consideration of the genotype of the mother by using a two-way ANOVA with the factors sub-strain of the mother and sub-strain of the pup. Finally, a principal component analysis with varimax rotation using the Kaiser criterion (eigen-values > 1) was calculated to examine patterns of relationships among call parameters. The exact p-values of 2-tailed testing were taken as measures of effect. A p-value of 0.05 was considered statistically significant. Data are shown as mean ± SEM.

3. Results

3.1. Within-strain embryo-transfer

Ultrasonic vocalization: Calling behavior differed considerably between B6JOla and B6N pups and was partly dependent on gender (see Fig. 1). Firstly, B6JOla pups emitted more calls than B6N pups (main effect pup: $F_{1,74}$=5.664, p=.020), irrespective of gender (main effect gender: $F_{1,74}$=0.886, p=.350; interaction pup x gender: $F_{1,74}$=0.470, p=.495). On the other hand, total calling time (main effect pup: $F_{1,74}$=2.157, p=.146) and mean call duration (main effect pup: $F_{1,74}$=1.590, p=.211) did not differ between the sub-strains, whereas females generally spent more time calling than males (main effect gender: $F_{1,74}$=4.155, p=.045; interaction pup x gender: $F_{1,74}$=1.710, p=.195). This effect was based on a difference in mean call duration, since female calls were longer than male calls (main effect gender: $F_{1,74}$=7.012, p=.010; interaction pup x gender: $F_{1,74}$=1.170, p=.283).

B6JOla and B6N pups also differed with respect to mean peak frequency and mean peak amplitude, since calls emitted by B6JOla pups were higher in frequency and amplitude (main effect pup: $F_{1,74}$=7.289, p=.009 and $F_{1,74}$=18.899, p<.001, respectively), whereas gender had no effect (main effect gender: $F_{1,74}$=0.004, p=.947 and $F_{1,74}$=0.776, p=.381, respectively; interaction pup x gender: $F_{1,74}$=0.204, p=.653 and $F_{1,74}$=1.252, p=.267, respectively). Finally, frequency modulation was higher in females (main effect gender: $F_{1,74}$=9.429, p=.003), but did not differ between sub-strains (main effect pup: $F_{1,74}$=1.418, p=.238; interaction pup x gender: $F_{1,74}$=2.685, p=.106).

Retrieval: B6JOla and B6N mothers did not differ significantly in the retrieval test, i.e. there were no differences in the latency to pick up (B6JOla: 18.83±8.65 s and B6N: 31.57±7.03 s; T_{11}=1.156, p=.272) or to retrieve the first pup (B6JOla: 46.00±18.61 s and B6N: 37.43±8.88 s; T_{11}=-0.436, p=.671).

3.2. Between-strain embryo-transfer

Ultrasonic vocalization: When cross-fostered pups were added to the analysis, results indicate that certain call parameters were primarily dependent on maternal effects, whereas others were primarily dependent on genotype or gender of the pup (see Table 1 and Fig. 2). Thus, the finding that B6JOla emitted more calls than B6N was based on maternal effects (main effect mother: $F_{1,106}$=4.457, p=.037), whereas pup genotype did not directly contribute to the observed difference (main effect pup: $F_{1,106}$=0.583, p=.447). Additionally, an interaction between mother and pup genotypes was observed (interaction mother x pup: $F_{1,106}$=11.733, p=.001), since pups raised by females of the same sub-strain emitted higher rates of ultrasonic calls in comparison to pups raised by females of the other sub-strain. This was especially true for B6JOla pups, since their calling behavior was highly affected by the sub-strain of the mother, whereas calling behavior of B6N pups was less affected by this factor. Remarkably, these effects were evident throughout testing (see Fig. 3). Gender did not directly or indirectly influence call number (main effect gender: $F_{1,106}$=0.005, p=.944; interaction mother x gender: $F_{1,106}$=0.803, p=.372; interaction pup x gender: $F_{1,106}$=0.005, p=.946; interaction mother x pup x gender: $F_{1,106}$=1.457, p=.230).

A similar picture was obtained for total calling time. Thus, the genotype of the mother affected the time spent calling (main effect mother: $F_{1,106}$=3.519, p=.063), whereas the genotype of the pup did not directly affect total calling time (main effect pup: $F_{1,106}$=013, p=.910). As for call number, however, total calling time was primarily dependent on an interaction between mother and pup sub-strain, since pups reared by mothers of the same sub-strain spent a longer time calling than pups reared by the other sub-strain (interaction mother x pup: $F_{1,106}$=6.121, p=.015). Gender had no effect on total calling time (main effect gender: $F_{1,106}$=0.725, p=.396; all p-values for interactions p>.100). Mean call duration was independent from genetic background, maternal care, and gender (main effect mother: $F_{1,106}$=0.001, p=.971; main effect pup: $F_{1,106}$=2.043, p=.156; main effect gender: $F_{1,106}$=1.871, p=.174; all p-values for interactions p>.100).

The mean peak frequency was dependent on pup genotype only, since B6JOla pups emitted calls with a higher mean peak frequency than B6N (main effect pup: $F_{1,106}$=7.810, p=.006), irrespective of the genotype of the mother (main effect mother: $F_{1,106}$=0.049, p=.824), or pup gender (main effect gender: $F_{1,106}$=0.005, p=.944). No significant interactions were observed (all p-values >.100). Conversely, mean peak amplitude was fully dependent on maternal effects. Pups reared by B6JOla emitted calls with a higher amplitude than pups reared by B6N (main effect mother: $F_{1,106}$=9.433, p=.003). Genotype of the pup and gender had virtually no influence on call amplitude (main effect pup: $F_{1,106}$=2.596, p=.110; main effect gender: $F_{1,106}$=1.205, p=.275; interaction mother x pup: $F_{1,106}$=0.519, p=.473; interaction mother x gender: $F_{1,106}$=0.005, p=.943; interaction pup x gender: $F_{1,106}$=1.632, p=.204; interaction mother x pup x gender: $F_{1,106}$=4.187, p=.043). Finally, frequency modulation was not dependent on the genotype of the mother (main effect mother: $F_{1,106}$=2.300, p=.132), but on the genotype of the pup (main effect pup: $F_{1,106}$=8.209, p=.005) and its gender (main effect gender: $F_{1,106}$=7.148, p=.009). Calls emitted by females were more modulated than those of males and calls emitted by B6N were more modulated than those of B6JOla. No significant interactions were observed (all p-values >.100). In short, the findings show that call amplitude is solely dependent on maternal effects, whereas call frequency and frequency modulation are solely dependent on the pup, i.e. its genotype and gender.

Despite differences in call rate and call features between both sub-strains, individual relationships between call parameters were similar as indicated by factor analyses (see Table 2). Thus, factor analyses revealed two dimensions in all four groups. Remarkably, in all four groups the first dimension was characterized by high positive factor loadings of call duration and frequency modulation, whereas the second dimension was characterized by a high positive factor loading of peak amplitude, but a high negative factor loading of peak frequency.

Furthermore, when analyzing calling behavior of animals with different background, no evidence for qualitative differences in their calling repertoire was obtained (see Fig. 4 and Fig. 5). Thus, although the scatter plots clearly indicate that the infant mouse calling repertoire contains different call types, the scatter plots show a profound overlap between groups. This means that both, prenatal cross-fostered and non-cross-fostered animals, show call types with an upper peak frequency ranging around 85–105 kHz and a lower peak frequency ranging around 60–70 kHz as a call type which was strongly frequency-modulated, i.e. showing a frequency modulation of about 40–60 kHz, and another one which was less frequency-modulated, i.e. showing a frequency modulation of about 0–20 kHz.

Retrieval: Again, no evidence for a difference in retrieval behavior between B6N and B6JOla mothers was obtained, i.e. no differences in the latency to pick up or retrieve the first pup were observed ($F_{1,15}$=1.615, p=.223 and $F_{1,15}$=0.200, p=.661, respectively). However, pup genotype affected the latency to pick up the first pup, since B6JOla were picked up sooner than B6N ($F_{1,15}$=5.127, p=.039). However, pup genotype did not affect the actual latency to retrieve the first pup ($F_{1,15}$=0.018, p=.894), and no significant interactions were obtained for the latency to pick up or retrieve the first pup (interaction mother x pup: $F_{1,15}$=2.464, p=.137 and $F_{1,15}$=2.300, p=.150, respectively). However, it is striking that the picture of the retrieval behavior appears to be inverse to the picture of call number (see Fig. 6).

4. Discussion

4.1. Comparison between B6JOla and B6N

The present results show for the first time that two sub-strains of C57BL/6 mice, namely B6JOla and B6N, differ in their ultrasonic calling behavior when isolated from dam and litter. This is in accordance with a bulk of observations of strain differences in the emission of ultrasonic vocalizations in mice (Bell et al., 1972; Cohen-Salmon et al., 1985; Hahn et al., 1987; Hahn et al., 1997; Hahn et al., 1998; Hahn & Schanz, 2002; Hennessy et al., 1980; Robinson & D'Udine, 1982; Roubertoux et al., 1996; Sales & Smith, 1978; Thornton et al., 2005), and adds to other differences between B6JOla and B6N.

Firstly, B6JOla and B6N mice differ genetically, since B6JOla mice carry a spontaneous deletion on chromosome 6 (Chen et al., 2002; Siegmund et al., 2005; Specht & Schoepfer, 2001; Specht & Schoepfer, 2004). This deficit leads to a loss of alpha-synuclein, a presynaptically localized protein that has been implicated in the etiology of Parkinson's disease (Maries et al., 2003, Polymeropoulos et al., 1997). Alpha-synuclein may have affected call production in infancy, possibly through its regulative function on dopaminergic transmission (Abeliovich et al., 2000; Oksman et al., 2006), since dopaminergic transmission itself influences ultrasonic calling in isolation (Cuomo et al., 1987; Dastur et al., 1999). However, the present gene-dependent findings cannot necessarily be attributed to alpha-synuclein deficits, since other genetic factors may have been critical or may have contributed. Indeed, detailed mapping and sequencing of the breakpoint recently revealed the absence of *Mmrn1* gene in addition (Specht & Schoepfer, 2004). A role of *Mmrn1* for ultrasonic calling is currently unknown.

Secondly, B6JOla and B6N mice differ in their adult anxiety-related behavior, namely the course of extinction of conditioned fear. Thus, B6JOla mice display lower levels of freezing to the context where they have been shocked before and shorter maximal fear responses (Radulovic et al., 1998; Siegmund et al., 2005; Siegmund & Wotjak, 2007; Stiedl et

al., 1999). Such behavioral differences are usually explained by genetic differences between strains. However, Siegmund et al. (2005) have shown that the difference in the extinction of fear memory in B6JOla and B6N is unlikely to be based on the different expression of alpha-synuclein. Therefore, it can be assumed that environmental factors contribute to such differences as well. Indeed, such factors have proven to hold strong epigenetic influence on the development of emotionality (Calatayud et al., 2001; Calatayud et al., 2004; Francis et al., 2003; for review see: Gordon & Hen, 2004) and out of these, maternal care is a crucial one (Caldji et al., 1998; Francis et al., 1999; Menard et al., 2004; Menard et al., 2007; Wöhr & Schwarting, in press; Zhang et al., 2005).

Finally, it can be noted that the virtual absence of gender differences in infant mice calling is in accordance with the vast majority of the literature (Hahn et al., 1997; Hahn et al., 2000; Hahn & Schanz, 2002; Roubertoux et al., 1996; but see: Hahn et al. 1998).

4.2. Nature versus nurture

By means of embryo-transfers, the present study demonstrates that the strain difference in the amount of ultrasonic calling is strongly dependent on the dyadic interaction between mother and pup. In contrast, most of the call features were primarily dependent on the pup itself. Thus, call frequency and frequency modulation were solely dependent on pup genotype and gender. There was one exception, however, namely amplitude, which was determined by the genotype of the mother. Finally, it is worth to note that the individual relationship between call parameters was similar in both sub-strains and that no differences in calling repertoire were observed.

Overall, the present findings are in line with studies of successful selective breeding for high or low calling rates in isolation (Brunelli, 2005, Brunelli et al., 2002, Brunelli et al., 2001, Brunelli et al., 1997, Hofer et al., 2001). Also, genetic analyses using reciprocal hybrids (Hahn et al., 1987; Hahn et al., 1997; Hahn et al., 1998; Hahn & Schanz, 2002; Roubertoux et

al., 1996; Thornton et al., 2005) revealed an influence of the genetic background on ultrasonic call emission; a finding, which is supported by studies on knockout mice. For instance, it was shown that several genes are involved in the production of ultrasonic vocalizations, especially *Foxp2* (Shu et al., 2005). Disruption of this gene led to a loss of ultrasonic vocalizations. Interestingly, *Foxp2* has been considered as a potential susceptibility locus for language disorders in humans (Lai et al., 2001).

However, genetic analyses also indicated maternal effects on call rate, duration, frequency, and frequency modulation (Roubertoux et al., 1996; Thornton et al., 2005). Actually, high levels of variability in call production were found even within lines selectively bred for high or low calling rates in isolation. For instance, Brunelli et al. (1997) observed that call rates ranged between 0 and 700 per min in the line selected for high rates of calling. Thus, it seems likely that epigenetic factors hold strong influence on isolation-induced calling, and the results of the present embryo-transfer support this assumption.

The finding that early environmental factors can influence calling behavior is in accordance with studies on the effects of prenatal malnutrition (Tonkiss et al., 2003), prenatal stress (Morgan et al., 1999; Williams et al., 1998), perinatal asphyxia (Calmandrei et al., 2004) or pre- and postnatal exposure of various substances, like alcohol (Barron et al., 2005; Marino et al., 2002; Tattoli et al., 2001), cocaine (Hahn et al., 2000), lead (De Marco et al., 2005), aluminum (Alleva et al., 1998), or carbon monoxide (Di Giovanni et al., 1993) on ultrasonic calling in infant rodents. However, in the natural context, variations in maternal care might be of major importance. This is indicated by studies on the effects of handling (Bell et al., 1971), maternal separation (D'Amato & Cabib, 1987; Zimmerberg et al., 2003a; Zimmerberg et al., 2003b), litter size (Hofer et al., 1993), and adoptions (Darnaudery et al., 2004) on ultrasonic calling in infant rodents. Darnaudery et al. (2004) found that pups raised by fostering dams showed less isolation-induced calling when compared to pups raised by their actual mothers, a finding which is similar to the present observation of lowered calling

behavior in prenatal cross-fostered pups. Remarkably, they also observed that this difference in call production was paralleled by a difference in maternal care, namely that fostering dams showed more maternal care than actual mothers, indicating that maternal care can reduce isolation-induced calling. Other evidence that maternal care can tune calling behavior in offspring was provided by D`Amato et al. (2005), who found that mouse pups raised by mothers with higher maternal responsiveness emitted lower call rates than pups of mothers with a comparatively low maternal responsiveness. Furthermore, Wöhr and Schwarting (in press) have shown that rat pups raised by mothers that demonstrated pronounced approach behavior in response to playback of isolation-induced calls called less in isolation than pups raised by mothers with weak or no approach behavior. Further, it was found that maternal licking is strongly linked to isolation-induced infant calling. Thus, rat pups that experienced a comparatively high rate of maternal licking emitted less calls in isolation than pups that were licked less often. A detailed analysis of ultrasonic calls revealed that apart from call number, several call features were affected by maternal care; and it is striking to see in the present mouse study that the call parameters affected by early environmental factors are quite similar to those, which were most predominantly influenced by maternal care in rats, namely call number and peak amplitude, but not peak frequency (Wöhr & Schwarting, in press). Changes in these call parameters might be of great functional relevance, since call rate, peak amplitude, and variability of calls, e.g. frequency modulation, are assumed to be a primary source of arousal induction in the mother (Ehret, 2005). Especially, the modulation of peak amplitude is interesting, because it was demonstrated that call amplitude can be reduced by anxiolytic drugs (Insel et al., 1986), and in adult rats it was shown that the averseness of the situation is encoded not only in call number but also in peak amplitude (Wöhr et al., 2005). Furthermore, peak amplitude was shown to be a valid predictor of the susceptibility to develop PTSD-like symptoms in response to a traumatic event in adulthood in the B6N sub-strain (Siegmund et al., unpublished observation).

However, the finding that early environmental factors, such as maternal care, are related to isolation-induced calling seems to contradict results of cross-fostering studies in rats (Brunelli et al., 2001) and mice (Hennessy et al., 1980), where no maternal effects on call rates were observed. With respect to the rat study by Brunelli et al. (2001) it has to be mentioned that they bred their animals for high or low calling rates by using a within-litter selection procedure which minimizes maternal effects (Hofer et al., 2001). Despite this selection procedure, however, Rojowsky et al. (2000) found that dams from the line with high calling rates showed reduced maternal responsiveness compared to dams from lines with random or low calling rates. With respect to the mouse study by Hennessy et al. (1980) it has to be noted that the authors reported that only one of the two strains used emitted ultrasonic calls, namely A/J, but not C57BL/6J. Bearing in mind the high call rates of B6N and B6JOla mice found in the present study, it seems likely that the absence of calls in the study of Hennessy et al. (1980) is based on the recording technology used there. They set their frequency tuner at 68 kHz with a bandwidth of 5 kHz, meaning that they were able to detect only a small proportion of calls according to the present findings. The present findings highlight the importance of using a sophisticated recording technology, which allows covering the frequency range from 50 up to 110 kHz. However, it might be also possible that maternal effects on ultrasonic calling behavior are only clearly evident when rectified pre- and postnatal experiences occur together. This would be in line with an embryo-transfer study in mice where it was shown that enhancing anxiety in otherwise low-anxious C57BL/6J pups requires both, pre- and postnatal experience with a more anxious dam (Francis et al., 2003). Whether maternal factors alone are sufficient for these differences to occur is currently evaluated by using reciprocal F1 hybrids.

The present finding that early environmental factors can affect isolation-induced ultrasonic calling is in line with a bulk of evidence showing that maternal factors strongly influence anxiety-related behavior in the offspring. Apart from the embryo-transfer study by

Francis et al. (2003), this was indicated in postnatal cross-fostering studies (Priebe et al., 2005; Zaharia et al., 1996) and reciprocal breeding of inbred mouse strains (Calatayud et al., 2001; Calatayud et al., 2004). Using backcrosses of hybrids from BALB/c and C57BL/6, i.e. using genetically identical pups which were exposed to different mothering styles, Calatayud et al. (2004) were able to verify their previous finding that maternal care can affect emotional reactivity as measured in the elevated plus maze and a free exploration paradigm. From rat studies, it is known that variations in maternal licking particularly affect the development of stable individual differences in emotionality. Thus, rats licked more often by mothers, showed decreased startle responses (Zhang et al., 2005), increased open field exploration (Caldji et al., 1998; Francis et al., 1999), shorter latencies to eat food provided in a novel environment (Caldji et al., 1998), fewer defensive responses in a resident-intruder test, and less shock-induced freezing (Menard et al., 2004; Menard et al., 2007) in adulthood than rats that were licked less often. Interestingly, these behavioral differences are accompanied by alternations in physiological stress reactivity (Liu et al., 1997) and various neural changes in brain areas implicated in anxiety regulation (Caldji., 1998; Liu et al., 1997; for review see: Gordon & Hen, 2004).

In total, the results of the present embryo-transfer study show that apart from call number several other call parameters differ between the two sub-strains, and that these differences are partly due to early environmental factors, and partly based on the genetic background. Although the present retrieval data do not allow to satisfactorily answer the question whether such differences are functionally relevant, they indicate that ultrasonic calling is positively related to retrieval behavior, since pup genotype affected the latency to pick up the first pup, i.e. B6JOla pups which emitted high levels of calls in isolation were picked up sooner than B6N pups which emitted fewer calls, whereas the mothers of both sub-strains did not differ significantly in their retrieval performance. Furthermore, it is striking that the picture of retrieval behavior is inverse to the picture of call number. Thus, the present

findings are in line with studies, which demonstrated that isolation-induced infant calling can induce maternal search and retrieval behavior (Allin & Banks, 1972; Sewell, 1970; Smith, 1976; Smotherman et al., 1974; Wöhr & Schwarting, in press; for review see: Ehret, 2005). Nevertheless, a definite answer on the functional relevance of these differences can best be obtained by conducting a playback experiment, which provides opportunity to test the communicative impact of specific call parameters without confounding variables, like odor. A playback experiment would also allow testing whether the temporal sequencing and call types are of functional relevance, e.g. whether the different call types observed here convey different information. Playback studies have already shown that lactating mice can distinguish between different call types, and that they prefer certain call types over other if given the choice (Ehret, 1992; Ehret & Haak, 1982; Smith, 1976). Finally, in light of the present study it would be promising to use more sophisticated analyses of maternal care including assessment of nursing styles and licking behavior in order to reveal potential sources for the differences between B6JOla and B6N mice.

4.3. Conclusion

The results of the present embryo-transfer study show that epigenetic factors can tune calling behavior in mouse pups. This adds to several other examples, where it was shown that particularly maternal care holds strong influence on anxiety-related behavior in infancy and adulthood.

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Figure legends:

Fig. 1: Column graphs comparing call number, total calling time (s), call duration (ms), peak frequency (kHz), peak amplitude (dB), and frequency modulation (kHz) between B6N (black) and B6JOla (white) pups originating from within-strain embryo-transfers, separately for males (squares) and females (circles).

Fig. 2: Comparison of B6JOla pups raised either by B6JOla mothers (J>J) or by B6N mothers (J>N), and of B6N pups raised either by B6N mothers (N>N) or by B6JOla mothers (N>J) regarding call number, total calling time (s), call duration (ms), peak frequency (kHz), peak amplitude (dB), and frequency modulation (kHz). Given are means±SEM.

Fig. 3: Time courses of ultrasonic vocalization per minute in B6JOla pups (left) raised either by B6JOla mothers (white circles) or by B6N mothers (black circles), and in B6N pups (right) raised either by B6JOla mothers (white circles) or by B6N mothers (black circles). Given are means±SEM.

Fig. 4: Scatter plots depicting distribution of calls, plotted with respect to duration, peak frequency, peak amplitude, and frequency modulation. Each dot reflects a single call. Calls emitted by B6JOla pups which were raised by B6JOla mothers are given in black, whereas calls emitted by B6JOla pups which were raised by B6N mothers are given in red. A lower-cut-off-frequency of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB.

Fig. 5: Scatter plots depicting distribution of calls, plotted with respect to duration, peak frequency, peak amplitude, and frequency modulation. Each dot reflects a single call. Calls emitted by B6N pups which were raised by B6N mothers are given in black, whereas calls emitted by B6N pups which were raised by B6JOla mothers are given in red. A lower-cut-off-frequency of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB.

Fig. 6: The left graph represents the number of calls emitted dependent on pup genotype, i.e. B6N (black circles) and B6JOla (white circles), and mother genotype (same data as in Fig. 2). The right graph represents the mean retrieval latency dependent on pup genotype, i.e. B6N (black circles) and B6JOla (white circles), and mother genotype. Given are means±SEM.

Figures:

Fig. 1:

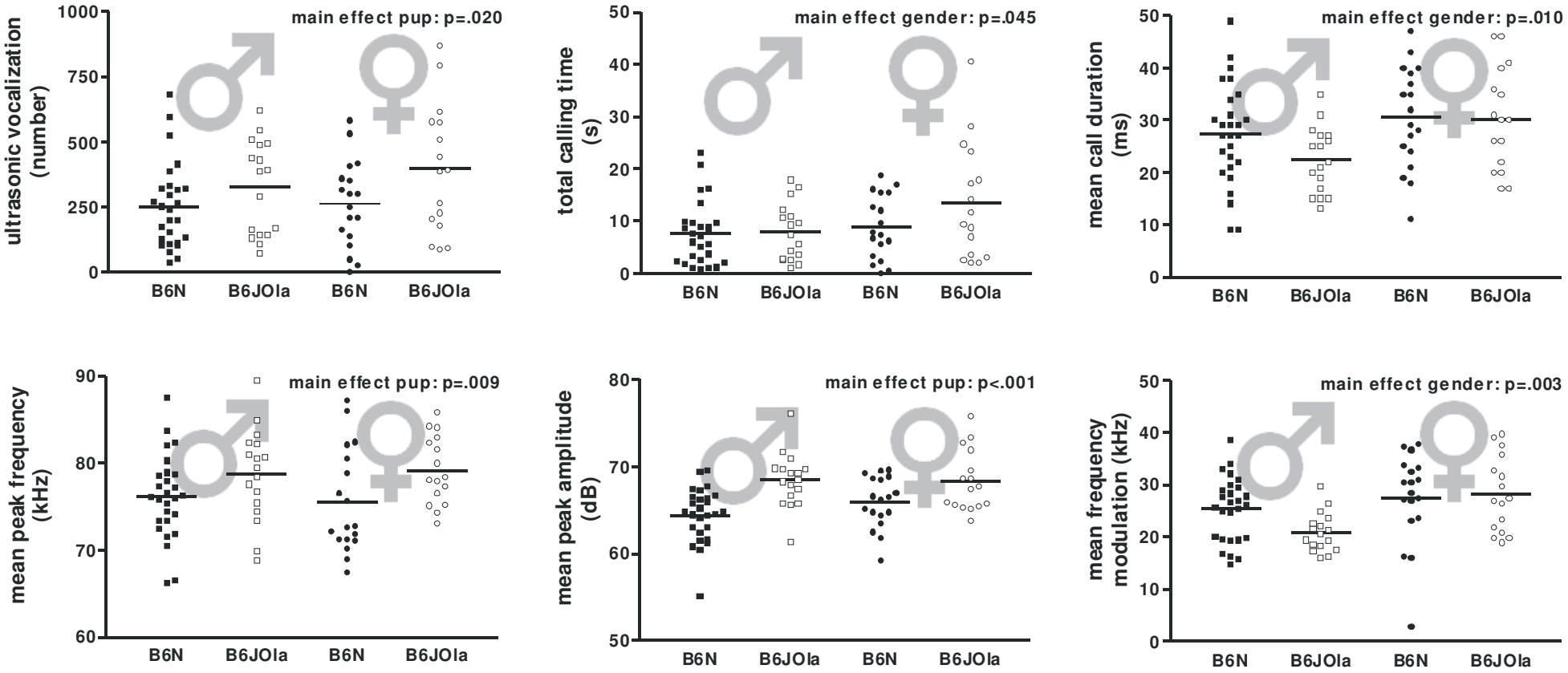


Fig. 2:

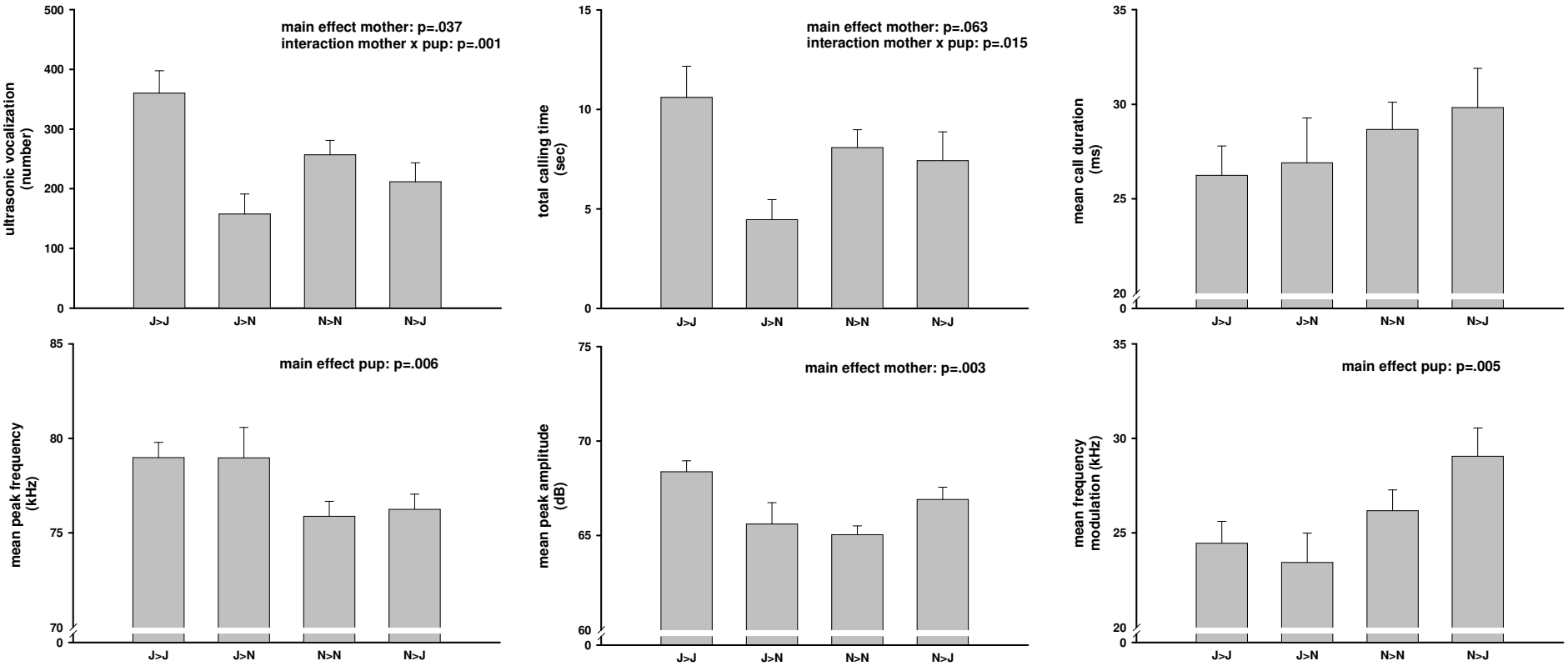

ultrasonic vocalization (number)
main effect mother: p=.037
interaction mother x pup: p=.001
total calling time (sec)
main effect mother: p=.063
interaction mother x pup: p=.015
mean call duration (ms)
mean peak frequency (kHz)
main effect pup: p=.006
mean peak amplitude (dB)
main effect mother: p=.003
mean frequency modulation (kHz)
main effect pup: p=.005
J>J
J>N
N>N
N>J

Fig. 3

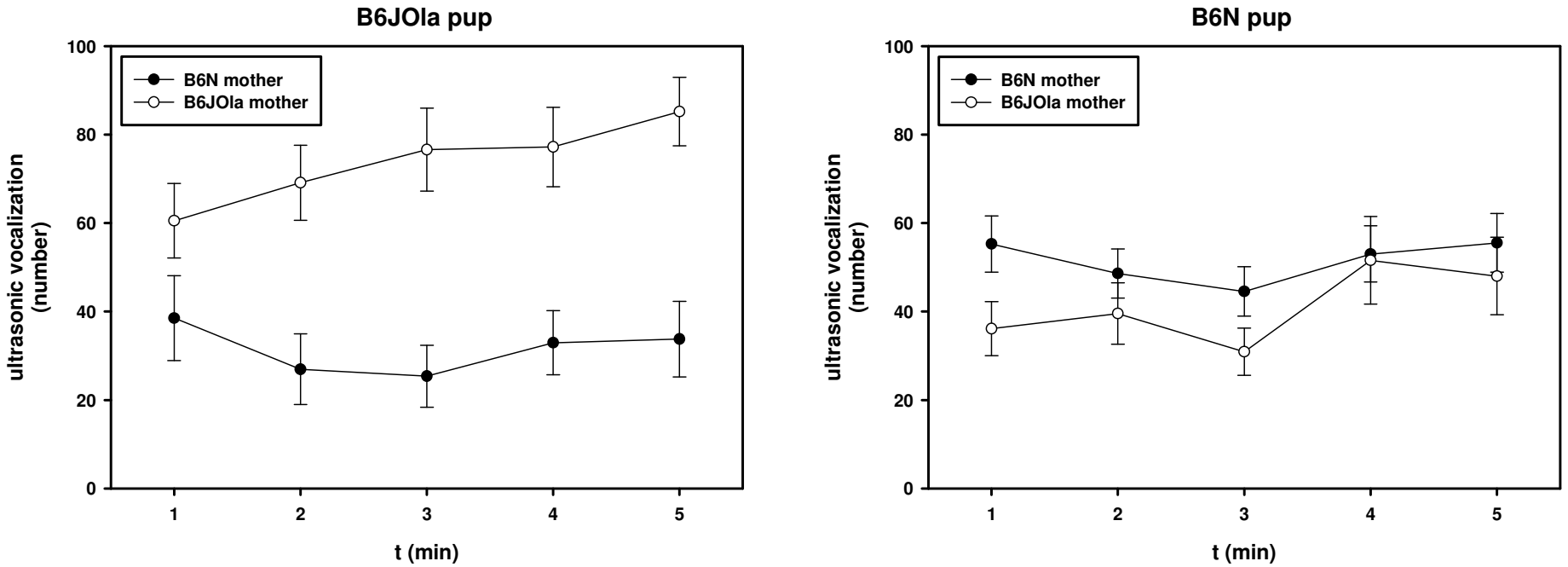

B6JOla pup
B6N mother
B6JOla mother
ultrasonic vocalization (number)
t (min)
0
20
40
60
80
100
1
2
3
4
5
B6N pup
B6N mother
B6JOla mother
ultrasonic vocalization (number)
t (min)

Fig. 4

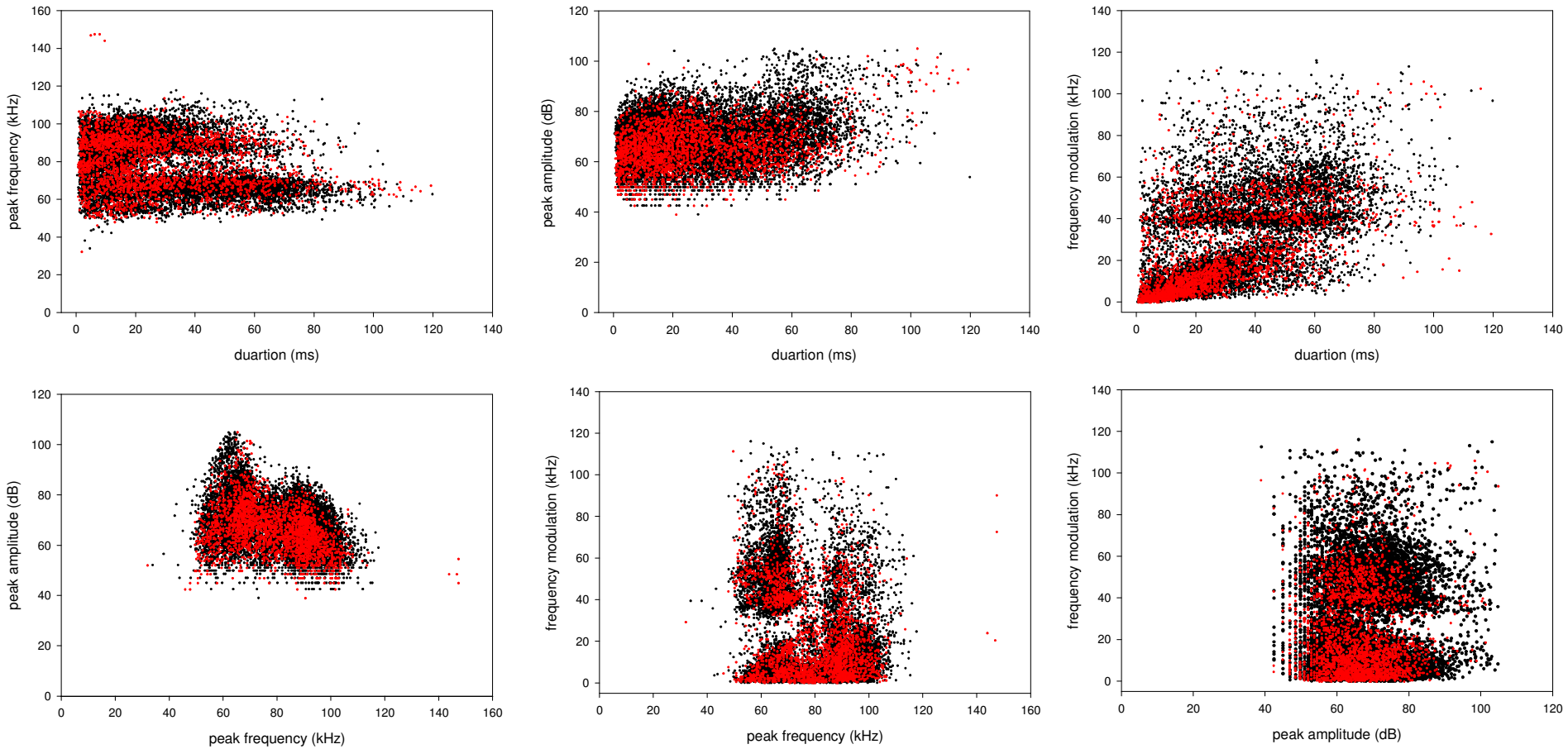

peak frequency (kHz)
duartion (ms)
peak amplitude (dB)
duartion (ms)
frequency modulation (kHz)
duartion (ms)
peak amplitude (dB)
peak frequency (kHz)
frequency modulation (kHz)
peak frequency (kHz)
frequency modulation (kHz)
peak amplitude (dB)

Fig. 5

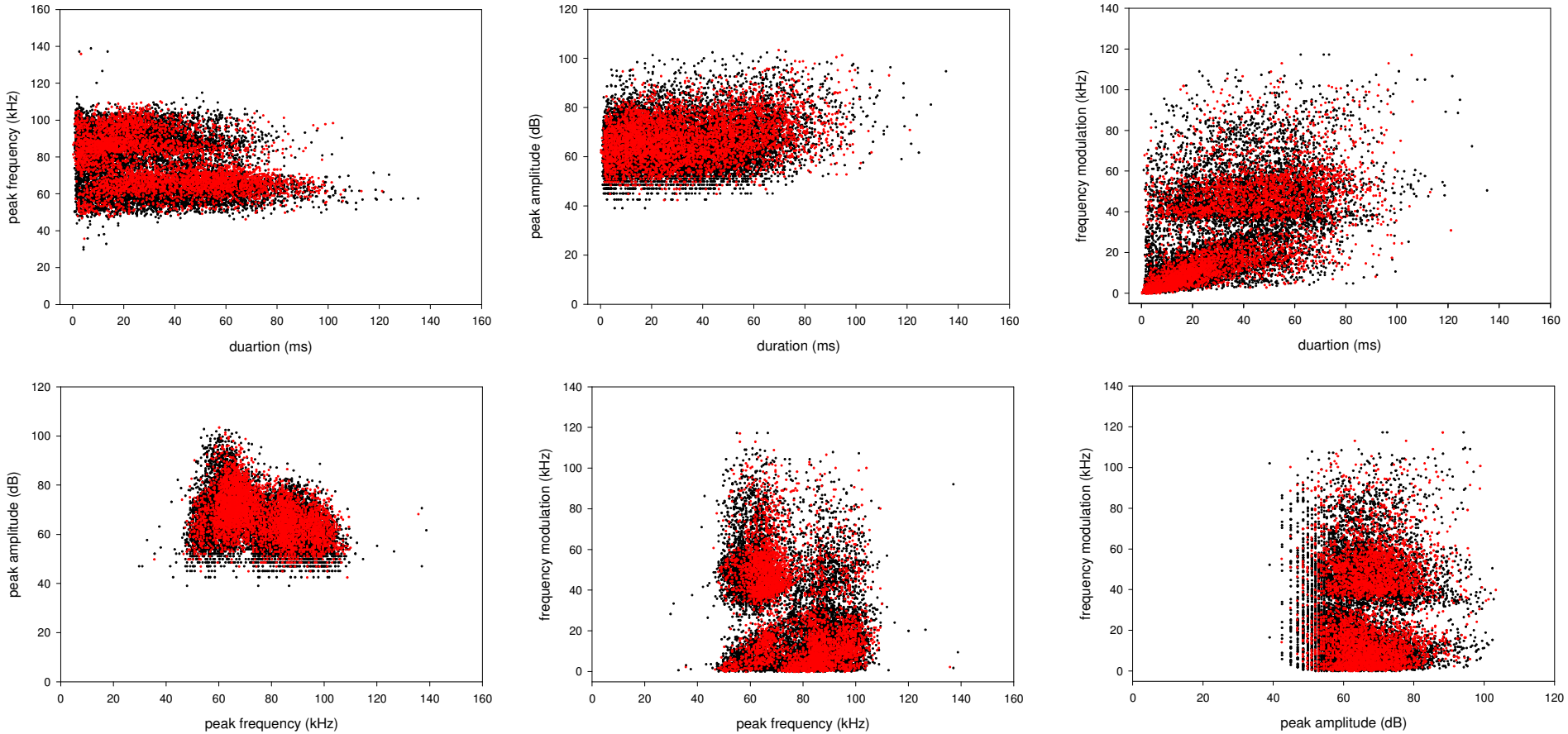
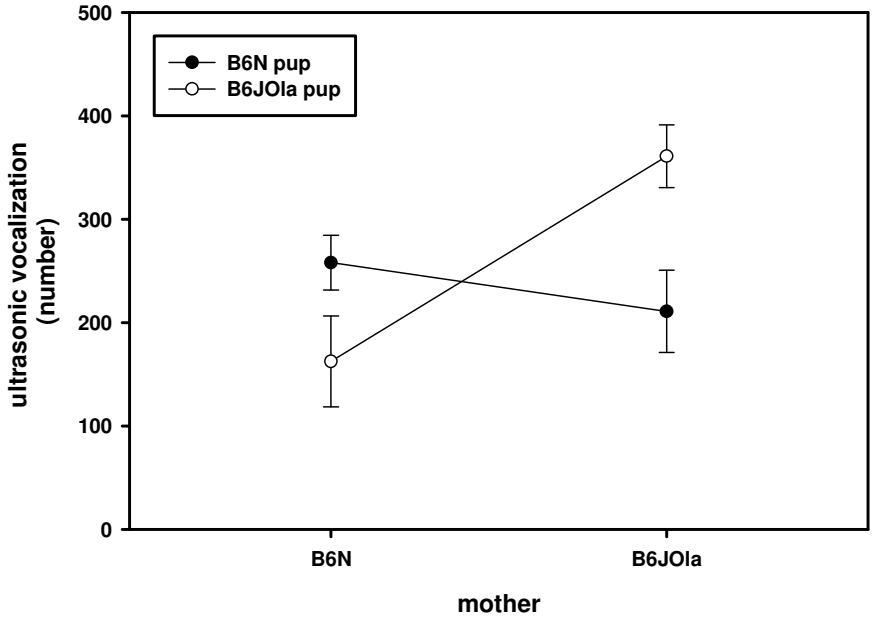
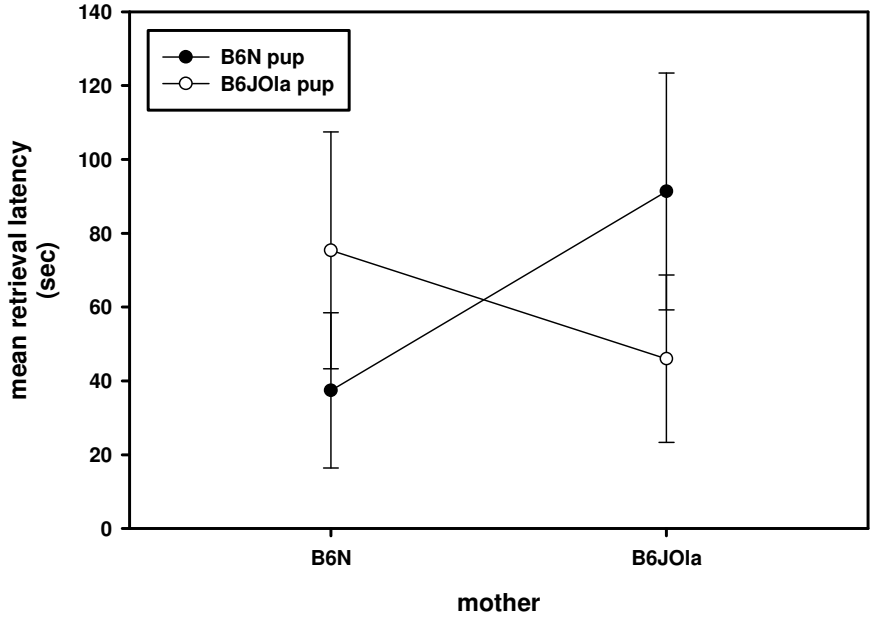

peak frequency (kHz)
duartion (ms)
peak amplitude (dB)
duration (ms)
frequency modulation (kHz)
duartion (ms)
peak amplitude (dB)
peak frequency (kHz)
frequency modulation (kHz)
peak frequency (kHz)
frequency modulation (kHz)
peak amplitude (dB)

Fig. 6


B6N pup
B6JOIa pup
ultrasonic vocalization (number)
500
400
300
200
100
0
B6N
B6JOIa
mother


B6N pup
B6JOIa pup
mean retrieval latency (sec)
140
120
100
80
60
40
20
0
B6N
B6JOIa
mother

Tables:

Table 1: Ultrasonic vocalization in B6JOla and B6N pups either raised by B6JOla or B6N mothers

		J>J	J>N	N>N	N>J
calls (n)	M	325.88±43.69	202.71±49.22	252.48±31.42	216.50±39.67
	F	396.38±61.84	122.34±45.28	263.56±39.07	205.25±55.57
total calling time (s)	M	7.86±1.33	5.34±1.21	7.59±1.17	7.64±1.78
	F	13.51±2.79	3.79±1.52	8.82±1.44	7.09±2.59
call duration (ms)	M	22.43±1.54	25.92±4.73	27.35±1.85	30.81±2.72
	F	30.26±2.44	27.67±2.35	30.63±2.31	28.34±3.37
peak frequency (kHz)	M	78.75±1.29	78.81±2.27	76.12±0.93	76.17±1.01
	F	79.20±0.99	79.01±2.35	75.51±1.41	76.34±1.38
call amplitude (dB)	M	68.46±0,77	67.32±1.42	64.45±0.60	67.39±0.83
	F	68.28±0.87	64.26±1.59	65.91±.0.69	66.17±1.04
frequency modulation (kHz)	M	20.91±0.90	21.34±2.57	25.29±1.19	28.50±1.81
	F	28.20±1.80	25.06±1.86	27.51±2.07	29.89±2.71

Comparison between B6JOla pups raised either by B6JOla mothers (J>J) or by B6N mothers (J>N), and B6N pups raised either by B6N mothers (N>N) or by B6JOla mothers (N>J) regarding call number, total calling time (s), call duration (ms), peak frequency (kHz), peak amplitude (dB), and frequency modulation (kHz). M = males; F = females. Values reflect means±SEM.

Table 2: Factor analysis of ultrasonic vocalization in B6JOla and B6N pups either raised by B6JOla or B6N mothers

	J>J		J>N		N>N		N>J	
	1. dimension	2. dimension	1. dimension	2. dimension	1. dimension	2. dimension	1. dimension	2. dimension
call duration (ms)	.943	.222	.966	.181	.936	.238	.918	.211
peak frequency (kHz)	.006	-.919	.096	-.862	-.162	-.893	.093	-.911
call amplitude (dB)	.232	.892	.304	.789	.212	.882	.320	.858
frequency modulation (kHz)	.959	.009	.943	-.007	.952	.162	.915	-.029
Variance explained (%)	46.60	42.28	48.12	35.29	46.33	41.44	44.74	40.32

Factor analysis of ultrasonic vocalizations emitted by B6JOla pups either raised by B6JOla mothers (J>J) or B6N mothers (J>N), and B6N pups either raised by B6N mothers (N>N) or B6JOla mothers (N>J). Values in columns reflect factor loadings, which express the association of each variable to the dimension. Variance explained gives the percentage of variance in the entire data set accounted for by each dimension.

Studie III

Wöhr, M., Houx, B., Schwarting, R.K.W. & Spruijt, B. (2008). Effects of experience and context on 50-kHz vocalizations in rats. *Physiology & Behavior*, 93 (4-5), 766-776.

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Effects of experience and context on 50-kHz vocalizations in rats

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Abstract

Rats can emit ultrasonic 50-kHz vocalizations which are generally assumed to reflect the animals' positive emotional state. However, some aspects question the reliability of 50-kHz calls as indicators of positive affective states. Firstly, rats also emit them in novel environments containing scents of other rats, or even while being victims of intra-species aggression. Secondly, huge inter-individual variability in call production can be observed. The present two studies were conducted to further determine factors other than reward, which may influence or even induce calling. Experiment A showed that 50-kHz calls were emitted in relatively high numbers during short isolation in test cages, and, to a lesser extent, also during testing in an open field and an elevated plus maze. Despite inter-individual variability, calling behavior was individually stable over days and occurred irrespective of whether rats were tested in a cage with or without familiar rat scents. These data indicate that 50-kHz calling is not necessarily a response to the presence of pleasurable or social stimuli. Additionally, it was observed that call emission during isolation is strongly affected by prior experience. Rats that had been trained repeatedly in an appetitive discrimination task emitted only few calls during short isolation in test cages, whereas naïve rats emitted high numbers of 50-kHz calls which decreased over time. The most likely explanation is that rats call in response to separation from the cage mate, as the first group was trained before the recordings, while the naïve rats were recorded immediately after separation. This explanation was supported by Experiment B, which showed that the rats that remained alone in the home cage also called at 50 kHz after separation from the cage mates. In both experiments, most of the 50-kHz calls were not frequency modulated, which lend support for the suggestion that this subtype has a social-coordinating function. The present findings urge sophisticated spectrographic analysis of ultrasonic vocalizations and caution when interpreting 50-kHz vocalizations, since specific subtypes of these calls can occur in contexts that are not necessarily pleasurable to rats, and are affected by prior experience and huge individual differences.

Keywords: Ultrasonic vocalization (USV); Spectrographic analysis; Individuality; Motivation; Emotion; Reward; Anxiety; Activity; Communication

1. Introduction

Rats emit distinct types of ultrasonic vocalizations, which differ depending on the animal's age, its current state and environmental factors [1–3]. Juvenile and adult rats mainly produce two different types of ultrasonic vocalizations, representing distinct affective states.

Low frequency vocalizations, often termed 22-kHz calls, are emitted when rats are exposed to predators [4], foot shocks [5–7], during inter-male aggression [8], drug withdrawal [9], handling [10], and social isolation [11]. Furthermore, anxiolytic drugs can reduce such vocalizations [12,13]. Accordingly, it was assumed that 22-kHz calls reflect a negative affective state akin to anxiety and sadness [5,6].

Conversely, high-frequency vocalizations, often termed 50-kHz calls, occur during or in anticipation of juvenile play [14,15], tickling [16–19], mating [20–24], food consumption [25], electrical self-stimulation of the brain [25], and addictive drugs [26–29]. On the other hand, aversive stimuli like cat scents [2], bright light [15], and the presence of a foot shock cue [25] can inhibit 50-kHz calling in otherwise rewarding situations. Recently, detailed spectrographic analysis of 50-kHz calls has revealed two call subtypes, of which predominantly the frequency modulated variety is emitted during tickling [30]. Remarkably, this call type resembles the squirrel monkey trill call which is related to appetitive behavior [31]. Based on a bulk

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of evidence, it was suggested that 50-kHz calls may serve as sensitive markers of unconditioned and conditioned states of reward [2], representing a rat homologue of human joy [18].

There is other experimental evidence, however, which shows that 50-kHz calls also occur in situations that are not necessarily appetitive to rats. For instance, 50-kHz calls were detected in various experimental controls, for example, in naïve rats that were placed into a test arena containing fresh bedding [24,32], or in saline-injected rats in drug studies [27–29]. Most intriguingly, rats emit 50-kHz calls during aggressive encounters such as in resident–intruder tests [8,13,33–39], with devocalization studies implicating the intruder as the source of high-frequency calls [40,41]. Rats even emit bursts of 50-kHz calls when entering an area associated with the potential presence of an offender [36,37]. Interestingly, the number of 50-kHz calls emitted by the intruder increased with the number of aggressive encounters in this context [37], which could be effectively decreased by anxiolytics, like diazepam [36]. 50-kHz calls have also been recorded when animals were restrained in positions resembling submissive postures [42]. Finally, rats undergoing morphine-withdrawal emit high-frequency calls [43]. All these results indicate that 50-kHz calls can occur also in non-rewarding or even aversive contexts. Indeed, Berridge [44] has questioned the interpretation that 50-kHz calls represent a rat homolog for human joy.

Secondly, a huge inter-individual variability in call production has been observed repeatedly [15,16,19,29]. Recently, Burgdorf and Panksepp ([45], p. 180) reported that only half of their adult rats show "reasonably high levels of tickle induced 50-kHz ultrasonic vocalizations but the remaining half remain very unresponsive". Schwarting et al. [19] provided evidence that such a variability results at least in part from dispositions or traits that are characteristic to the subject under study. Apart from these studies, variability in the emission of 50-kHz calls has received little specific attention, except, that the propensity to emit tickling-induced 50-kHz calls was used to breed rat lines with high and low call rates [17,46].

The present two studies were conducted mainly to determine which contexts and factors other than reward may influence or even induce calling. In Experiment A, calling behavior during solitary exposure to a novel cage was compared between naïve rats versus rats that had prior experience with a food-reinforced discrimination task. It was expected that the latter, due to their experience of an appetitive testing situation, would be less anxious and therefore emit more calls during the housing cage test than their naïve cage mates. In order to screen for potential individual differences in calling, the rats were recorded on three consecutive days. Recording was done in cages with scents of other rats in order to assure high calling rates [32]. The effect of the appetitive and social value of the additional rat scents was tested on the second day, when half of the rats were placed in clean cages without rat scents. In line with Brudzynski and Pniak [32], it was expected that the absence of scents would reduce call rate, and affect qualitative aspects of calling. Finally, in order to identify behavioral measures that might correlate with ultrasonic calling, rats were tested in an open field to determine their reactivity to novelty and their anxiety on the elevated plus maze. These tests were applied since it was shown repeatedly that male Wistar rats, although identical in breeder, age and housing conditions, can show stable individual differences in their locomotor activity in response to novelty [47–51] and anxiety-related behavior [52–55]. As the results of Experiment A were difficult to explain by the affective hypothesis on 50-kHz calling, it was hypothesized alternatively that rats call for their mates, that is, that 50-kHz calls may also serve to establish or keep social contact. This expectation was tested in Experiment B.

2. Experiment A

2.1. Methods

2.1.1. Subjects and housing

Twenty-four naïve male Wistar rats (HsdCpb:WU, Harlan, The Netherlands), weighing 280–310 g on delivery, were used. They were housed in pairs in Makrolon type IV cages (1815 cm^2; height: 20 cm; Tecniplast, Milan, Italy) with a heightened metal wire lid. The cages, which contained bedding material type LTE E-001 (Abedd, Dominik Mayr KEG, Köflach, Austria), were enriched with a shelter, two gnawing sticks and paper towels. Lab chow (RMH-B®, Hope Farms, Woerden, The Netherlands) and water were available ad libitum. Animals were housed in temperature-controlled rooms (22 ± 1 °C) under a reversed dark/light cycle (dim red light; 50 lx: 7:00–19:00; bright white light; 285 lx: 19:00–7:00) with background music (56 dB) throughout. Cages were cleaned and the animals weighed once a week after the day's experimental tests.

2.1.2. General procedure

Here, two groups of rats were compared, namely experimentally naïve rats versus subjects which had repeatedly been tested in an appetitive discrimination task before being monitored for ultrasonic vocalization. Initially, all animals were allowed to adjust to the housing and light conditions for 5 days and were handled on two days during this period. Thereafter, one rat of each cage pair was trained daily in a T-maze discrimination task (termed: experienced rats, $n = 12$). The cage mate stayed in the home cage during this time (termed: naïve rats, $n = 12$). To enhance motivation in the T-maze, food in the home cage was removed in the early morning of each training day. Recordings of the ultrasonic vocalizations began three weeks after the start of training, when animals were approximately 12 weeks old (body weight: 355 ± 19 g). Experienced and naïve rats were recorded individually on three consecutive days (termed: housing cage test). Two weeks thereafter, training in the discrimination task ended. One week later, all animals were screened in an open field. Finally, they were tested in the elevated plus maze one day later. All training and testing was conducted within the dark phase between 9:00 and 16:00 h (without background music).

All experimental procedures were performed according to the legal requirements of The Netherlands, and had been approved by the Ethical Committee of the Utrecht University.

2.1.3. Appetitive discrimination task

A wooden T-maze was used which consisted of a start box ($l \times w \times h$: 30 × 25 × 30 cm) and two goal boxes (30 × 30 × 60 cm), connected by arms (70 × 30 × 60 cm; for details see: [56]). After two 10 min habituation sessions without reward, the daily training sessions started, each consisting of 10 trials and lasting about 30 min. In the first phase, the rats were trained to discriminate the arm that contained a saucer with syrup from the arm with an empty saucer. At the time of the first housing cage test (see below) rats had experienced 7 training sessions. After reaching a criterion of 8/10 correct choices per session, they entered a second phase of 4 sessions in which they had to discriminate between syrup with and without capsaicin. Only 7 rats reached this phase. The preference for syrup was assessed again in the third phase, which was similar to the first phase. By the end of the training, all rats had experienced between 8 and 21 sessions. A Makrolon type III cage (840 cm^2; height: 18 cm; Tecniplast) was used to transport rats to and from the T-maze apparatus.

2.1.4. Housing cage test

For ultrasonic recording, rats were tested individually in a separate experimental room. Recordings were made under dim red light (50 lx) in a Makrolon type III cage with a flat metal wire lid and bedding material type LTE E-001 (Abedd, Dominik Mayr KEG). Experienced rats were trained in the T-maze task before being placed into the recording cage, whereas naïve rats were immediately transferred to the cage. Both groups were tested on three consecutive days for 15 min. On the first day, the cages contained soiled bedding from the home cage, i.e. bedding with scents of both rats. On the second day, half of the animals of each group were exposed to a cage with fresh bedding, whereas the other half was exposed again to a soiled cage. On the third day, all rats were placed into soiled cages again. During testing, only one animal was present in the experimental room.

2.1.5. Open field test

This test was used to measure individual levels of locomotor activity and anxiety-related behavior. The apparatus, which consisted of a round arena (75 cm diameter) with a 33 cm high wall, was made of dark grey plastic and was located in the middle of a separate experimental room held under dim red light illumination (32 lx). Behavior was monitored with a video camera (Panasonic, NV DS29) suspended 260 cm above the open field. Before each trial, the apparatus was cleaned with warm water and soap, and then dried off with paper towels. Each animal was placed into the open field, facing the wall. Immediately thereafter, sound recording and the observation program (EthoVision; EVCP 3.0, Noldus Information Technology, Wageningen, The Netherlands) were started and the animal was observed for 5 min. Subjects were tested in a randomised order. For behavioral analysis, two concentric areas were defined using EthoVision: centre (25 cm diameter) and outer zone (25 cm width, measured from the wall). As measures of anxiety, latency to enter the centre and time spent in the centre were considered. An entry was defined as the animal's centre of gravity being within a specific area of the open field. Locomotion, that is, the total distance traveled in cm, and rearing behavior were considered as indices of activity. Rearing was measured by an experienced observer.

2.1.6. Elevated plus maze test

The plus-shaped apparatus was made of black plastic and consisted of two closed ($l \times w \times h$: 40 × 10 × 27 cm), two opposite open arms (40 × 10 cm), and a central square (30 × 30 cm). The maze was elevated 74 cm above the floor and was located in a separate room with bright white light illumination (260 lx). For behavioral recording, a video camera (Panasonic, NV DS29) was suspended 186 cm above the maze. Before each trial, the apparatus was cleaned as described above. Each animal was placed into the centre, facing one of the open arms. Immediately thereafter, sound recording and data collection with the EthoVision program began. Animals were observed for 5 min in a randomised order. Parameters recorded were the duration and frequency of entries to each of the five areas. To measure anxiety-related behavior, the total frequency of open arm entries and the total time spent on the open arms were measured, along with the latency to enter an open arm.

2.1.7. Ultrasonic recording and analysis

Rat calls were recorded using an UltraSoundGate Condenser Microphone CM16 (Avisoft Bioacoustics, Berlin, Germany), which was positioned 30 cm above the floor of the cage and 60 cm above the elevated plus maze and the centre of the open field. This microphone was sensitive to frequencies of 15–180 kHz, with a flat frequency response (±6 dB) between 25 and 140 kHz. It was connected via an UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a computer. Acoustic data were displayed in real time by the Avisoft RECORDER, a multi-channel triggering hard-disk recording software (version 2.95; Avisoft Bioacoustics), and were recorded at a sampling rate of 214,285 Hz in 16 bit format.

For acoustic analysis, recordings were transferred to Avisoft SASLab Pro (version 4.34; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100% Frame, Hamming window and 75% time window overlap). Accordingly, the spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. A lower-cut-off-frequency of 20 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Each call was manually marked by a section label to be included in the automated parameter measurement. Then, various parameters were determined automatically, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. As temporal parameters, latency to call, mean call duration, and total calling time were determined. Finally, the total number of calls emitted was measured. On the basis of their shape, calls were classified into flat and frequency modulated ones according to Burgdorf and Panksepp [45]. Calls containing both types (see Fig. 1c and d) were classified as

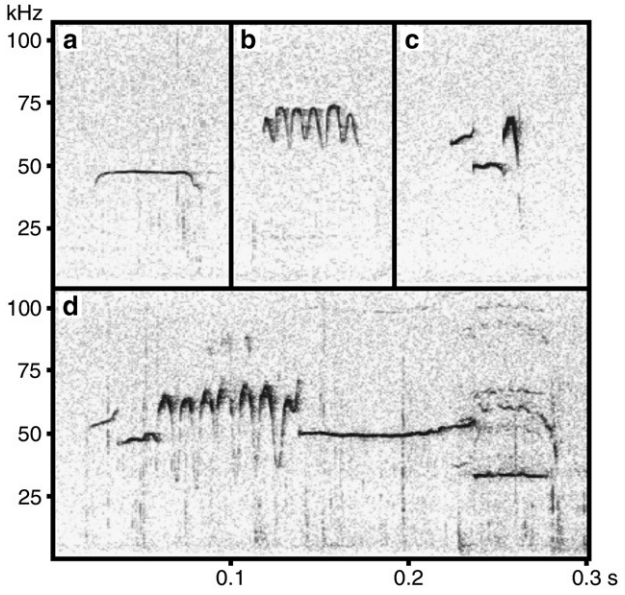

Fig. 1. Spectrograms of four exemplary calls emitted during the housing cage test. Part (a) shows a flat 50-kHz call with one element. Part (b) shows a frequency modulated 50-kHz call with one element. Part (c) shows a 50-kHz frequency step call with three elements. Elements one and two are flat, whereas element three is frequency modulated. Part (d) shows a 50-kHz call with five elements where only element three is frequency modulated.

frequency modulated. Furthermore, calls emitted during the first housing cage test were analysed in more detail by an experienced observer. Here, the extent of frequency modulation, i.e. the difference between the lowest and the highest peak frequency within each specific call, and the number of call elements were measured (see Fig. 1a–d).

2.1.8. Statistical analysis

For each subject, the mean of each call parameter served as the statistical unit. Unpaired two-tailed *t*-tests were used to determine whether experienced and naïve animals differ in call production during housing cage test. *p*-values were corrected for unequal variances when appropriate. An ANOVA for repeated measurements was used to test whether the time course of ultrasonic calling differed between both groups in the first housing cage test. Subsequent paired *t*-tests were used to compare call emission in the first minute versus the last minute. Due to the relatively small number of animals, Mann–Whitney *U*-tests were used to determine the effect of scents [57]. Mann–Whitney *U*-tests were also used for comparisons between experienced and naïve rats in calling and overt behavior during open field testing and elevated plus maze screening. Pearson's correlation coefficient was used to correlate call behavior over days and to explore the relationship between overt behavior and vocalization.

2.2. Results

2.2.1. Housing cage test

During the housing cage test, calls in the range of 22 kHz and 50 kHz occurred. Out of these, 22-kHz calls were emitted only rarely, that is, on the last two days of the housing cage test, one and two experienced animals, respectively, emitted 22-kHz calls. Naïve rats did not display 22-kHz calls. Predominantly, 50-kHz vocalizations were detected. These were characterized by high variability within and between subjects, especially with respect to their shapes (for examples see Fig. 1).

2.2.1.1. Preceding experiences. 50-kHz calling in the housing cage test was largely affected by preceding experiences (see Fig. 2). On the first day in the housing cage test, T-maze trained rats ($n=9$, since 3 rats had to be excluded due to partial data loss) vocalized with a mean rate of 12.79±4.62 calls, whereas naïve rats ($n=12$) produced 608.81±102.59 calls ($t_{11.045}=5.803$, $p<.001$). Although call rate gradually decreased over minutes ($F_{6,14}=14.091$, $p<.001$), the group difference was detectable at every time point ($F_{1,19}=25.546$, $p<.001$; subsequent *t*-tests: all *p*-values<.050; see Fig. 3). Also, the time course of call emission differed between both groups ($F_{6,14}=8.690$, $p<.001$). Subsequent *t*-tests showed that call rates declined from 76.87±17.53 calls during the first minute to 11.01±3.69 during the last in naïve animals ($t_{11}=3.949$, $p=.002$), whereas in experienced rats, the decrease from the first (2.90±1.63) to the last minute (0.32±0.24) was not significant ($t_7=1.793$, $p=.111$). Similar to call rate, total calling time was higher in naïve rats (20.91±5.56 s) than in experienced ones (0.29±0.13 s; $t_{11.012}=3,700$, $p=.003$).

Regarding call types, naïve rats emitted both, more flat ($t_{11.064}=6.015$, $p<.001$) and more frequency modulated calls ($t_{11.020}=4,993$, $p<.001$) than experienced rats. Notably, the ratio between flat and frequency modulated calls differed ($t_{11}=-5.362$, $p<.001$), since experienced rats emitted mostly flat calls (91.57±11.57%), as compared to 66.75±9.64% in naïve rats. The mean number of call elements was equal in both groups ($t_{9.748}=3.226$, $p=.681$), but call length was longer in naïve (31.09±2.54 ms) than experienced rats (19.03±2.68 ms; $t_{19}=3.226$, $p=.004$). Also, naïve rats started calling after 4.42±3.62 s as compared to 218.60±105.10 s in experienced rats ($t_{8.019}=-2.037$, $p=.076$).

Additional group differences were found in peak amplitude and peak frequency. Firstly, peak amplitude was lower in experienced (46.10±1.17 dB) than naïve rats (52.90±0.68 dB; $t_{19}=5.305$, $p<.001$). Secondly, calls uttered by experienced rats showed a mean peak frequency of 46.06±2.84 kHz, which was lower than that of naïve animals (55.53±1.06 kHz, $t_{19}=3.470$, $p=.003$). Finally, frequency modulation was less broad in experienced (5.89±1.03 kHz) than in naïve animals (9.73±0.86 kHz; $t_{19}=2.880$, $p=.010$).

On the second and third days, call rates of experienced rats remained low in the housing cage test (27.31±15.24 and 22.89±11.30 calls, respectively). These rats were therefore excluded from further analysis concerning the effect of scents and the stability of ultrasonic calling over days.

2.2.1.2. Scents. On the second day, the previously naïve rats were exposed singly to a cage which contained either familiar scents again or fresh bedding ($n=6$ each; Table 1). Apart from a difference in the very first minute ($U=5.0$, $p=.041$), animals

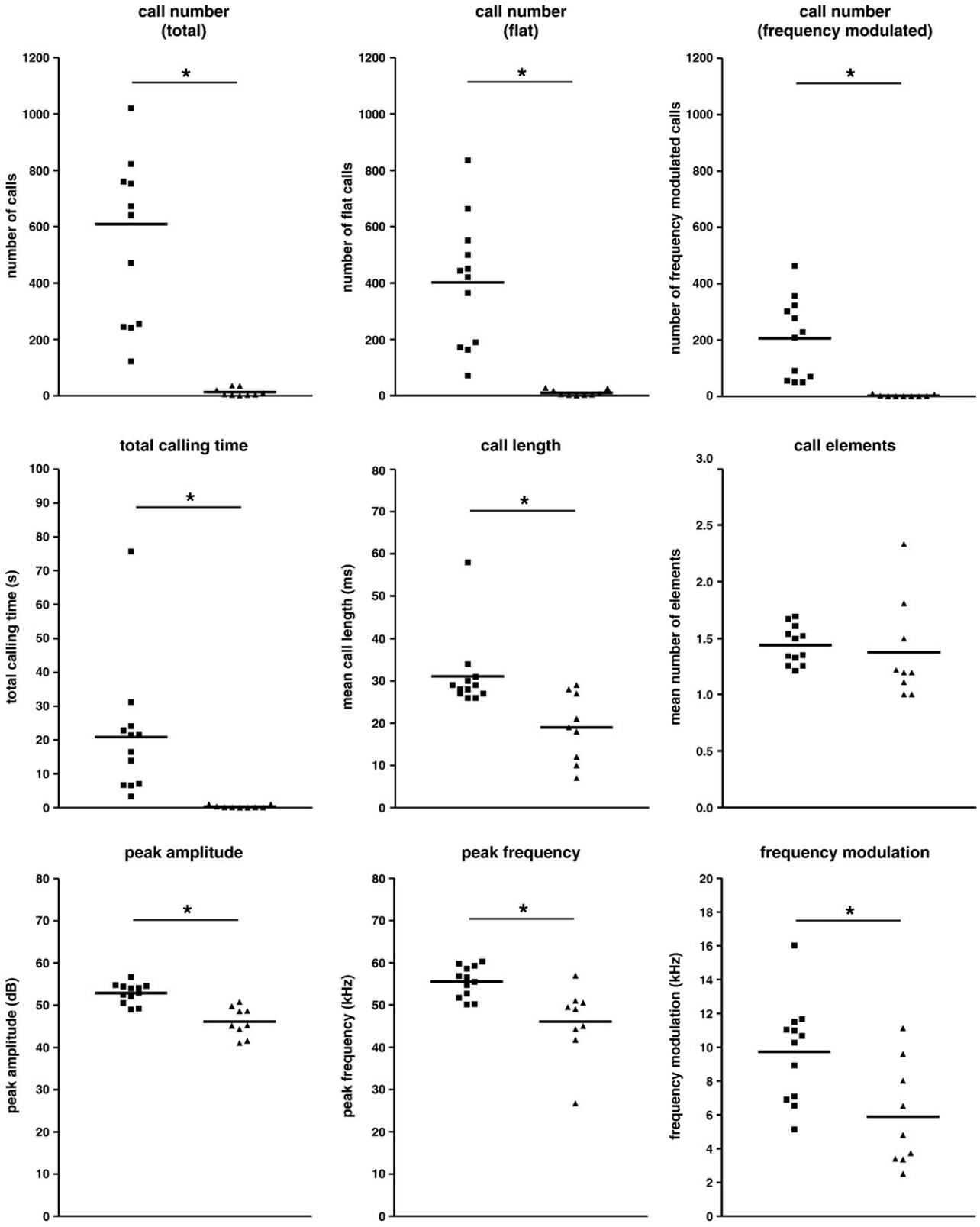

Fig. 2. Column graphs depicting the effect of prior experience on call number (total, flat, and frequency modulated), total calling time (s), call length (ms), number of call elements, peak amplitude (dB), peak frequency (kHz), and frequency modulation (kHz) during housing cage test 1. Symbols reflect individual averages (except for call number and total calling time) of rats which were naïve (squares; $n=12$) or experienced (triangles, $n=9$). Significant group differences are marked with asterisks: $^{*}p<.05$.

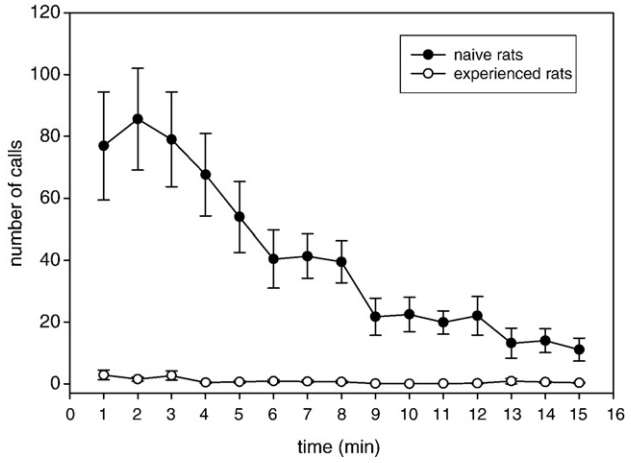

Fig. 3. Time course of call number per minute during housing cage test 1. Symbols reflect group averages for rats which were naïve (black circles; $n=12$), or experienced (open circles, $n=9$).

which were tested in a soiled cage did not emit more calls than animals tested in a clean cage ($U=11.0$, $p=.310$). Also, there were no differences in total calling time ($U=11.0$, $p=.310$), mean call duration ($U=14.0$, $p=.589$), amplitude ($U=15.0$, $p=.699$), peak frequency ($U=15.0$, $p=.699$), or latency to call ($U=7.0$, $p=.093$). An unexpected a-priori difference between both groups was detected on the first testing day, since animals, which were exposed to the soiled cage, had emitted significantly more calls on the first day of the housing cage test than animals which were exposed to the clean cage ($U=5.0$, $p=.041$).

2.2.1.3. Individuality. On day 3, the naïve animals were again tested in a cage containing fresh bedding. Here, they still emitted rather high rates of 50-kHz calls, although a reduction from day 1 to day 3 was observed (608.81±102.59 versus 358.01±58.98 calls; $t_{11}=5.933$, $p<.001$). Furthermore, call rate decreased over minutes in a pattern similar to that of day 1. Call rates did not differ between animals which had been either exposed to a cage containing familiar scents or fresh bedding the day before ($U=19.0$, $p=.699$).

Between days, stable individual differences in call emission were detected. For one, call rates were significantly correlated (day 1 versus 3: $r=.719$, $p=.008$). Similar correlations were obtained in case of call duration ($r=.970$, $<.001$) and total calling time ($r=.792$, $p=.002$), and a trend in case of latency to call ($r=.530$, $p=.076$). Apart from those temporal parameters, peak frequency ($r=.670$, $p=.017$) and amplitude ($r=.577$, $p=.050$) were also significantly correlated between days 1 and 3.

2.2.2. Open field test

Behavioral parameters in the novel open field were not affected by the preceding experience of T-maze training, since experienced and naïve animals did not differ in any behavioral parameter (all p-values$>.100$). Compared to the housing cage test, 50-kHz calls were detected only rarely. This time, however, naïve animals emitted less calls (2.17±0.93) than experienced ones (6.08±1.77; $U=35.0$, $p=.028$). This effect disappeared when only calling animals were considered for analysis ($U=17.0$, $p=.160$), since only 6 out of 12 naïve, but 10 of 12 experienced animals, vocalized. Overall, animals emitted mostly flat calls (63.66±8.68%). Despite the fact that groups did not differ in the relative number of frequency modulated and flat calls ($U=21.50$, $p=.192$), the higher proportion of flat calls was only evident in experienced rats (73.95±6.36%) and not in naïve rats (46.53±19.67%). Notably, none of them displayed 22-kHz calls.

Since naïve, but not experienced, rats had shown substantial call rates in the housing cage test, only the former were used to correlate calls in the housing cage test with behavior in the open field. These animals entered the centre 9.75±1.72 times and spent 13.90±2.34 s there. The first entry into the centre occurred after 32.48±12.08 s. Furthermore, the animals traveled a total of 2395.86±101.59 cm (centre: 164.10±27.24 cm) and showed 35.50±2.44 times of rearing. Pearson's correlation revealed that the distance moved in the open field was positively correlated with total calling time ($r=.582$, $p=.047$) and mean call length ($r=.608$, $p=.036$) in housing cage test 1. Also, a negative correlation with peak frequency was obtained ($r=-.719$, $p=.008$). None of the other parameters yielded substantial correlations (all p-values$>.100$).

2.2.3. Elevated plus maze test

Similar to the open field, no 22-kHz and only a few 50-kHz calls were recorded in the elevated plus maze. Again, experienced animals predominately emitted 50-kHz calls (experienced rats: 8.27±1.97; naïve rats: 3.08±0.98; $U=30.0$, $p=.025$). This effect was again largely due to call likelihood, since no difference ($U=30.0$, $p=.152$) was observed when only vocalizing animals were analysed (naïve: 9/12; experienced: 11/11 — one animal was excluded from further analysis since it fell off the plus maze). Remarkably, both groups emitted primarily flat calls (experienced rats: 79.79±5.36%; naïve rats: 62.84±13.77%; $U=38.50$, $p=.412$). Apart from ultrasonic calling, groups did not differ significantly in behavioral parameters (all p-values$>.100$). The number of calls emitted in the open field was positively correlated with that emitted in the plus maze ($r=.581$, $p=.004$).

Again, only naïve rats were used to compare vocalization in the housing cage test with plus maze behavior. These animals spent most of the time in the enclosed arms (249.65±7.55 s), entered the open arms rarely in comparison to enclosed arms (0.50±0.26 and 7.75±1.27, respectively), and first entered the open arm after 240.12±29.91 s. Plus maze behavior was only

Table 1
Comparison between rats placed in soiled and unsoiled cages

	Unsoiled ($n=6$)	Soiled ($n=6$)	
Latency to first call (s)	16.36±5.93	5.04±3.67	$p=.093$
Number of calls	256.50±67.83	379.17±70.78	$p=.310$
Number of calls in first min	30.50±18.96	74.67±18.69	$p=.041$
Total calling time (s)	7.71±1.86	13.06±3.33	$p=.310$
Mean call length (ms)	30.20±1.40	33.21±3.52	$p=.589$
Peak amplitude (dB)	51.45±1.41	50.86±0.83	$p=.699$
Peak frequency (kHz)	53.52±1.86	53.86±1.45	$p=.699$

Given are means±SEM. p-values reflect the results of Mann–Whitney U-test.

weakly associated with ultrasonic calling during the housing cage test, since only the latency to utter the first call was negatively correlated with the latency to enter an open arm ($r=-.726$, $p=.007$; all other p-values $>.100$).

3. Experiment B

Experiment A had shown that rats which were experienced in appetitive discrimination tasks barely vocalized, while naïve rats vocalized at surprisingly high levels. This indicates that preceding experience affects calling, although it remains unclear why. Apart from the training experience, the rats of both groups differed in another manner: the naïve rats were recorded immediately after separation from their cage mate, while the cage mate was recorded about 30 min later, as it was being trained first. The steep decline in calling rate of the naïve rats suggests that the motivation to call is strongest upon entering the test cage. An explanation for these findings could be that the rats call after separation from their cage mate to keep contact, and reduce calling when they get no response. If this hypothesis is true, then one would expect that rats which remain in the home cage also call after separation. This prediction is tested in Experiment B.

3.1. Methods

3.1.1. Subjects and housing

10 Wistar (HsdCpb:WU, Harlan, The Netherlands) and 16 Long-Evans (HsdBlu:LE, Harlan, The Netherlands) housed in groups of 2–3 in Makrolon type IV cages under the same conditions as in Experiment A were used. Since surplus rats that had been part of earlier experiments were used, the strains differed in age and experience at the time of recording.

Long-Evans rats were tested at the age of about 12 months. At about 2 months, they had learned to associate a sound with oncoming reward (for details see: [58]). During that period, which lasted 4–6 weeks, they were housed in either standard ($n=8$) or enriched ($n=8$) Makrolon IV cages comparable to the ones used in Experiment A. Until ultrasonic testing, the rats were handled regularly, but remained otherwise relatively undisturbed in their home cage.

Wistar rats were tested at an age of about 6 months. At about 2 months, they had been used for the training of aggressive rats that served as residents in a resident–intruder test. Each rat experienced aggressive resident behavior during 2–3 training sessions over a period of 2 weeks. They were housed like enriched Long-Evans rats, and were handled regularly.

3.1.2. Procedure

Two rats of the same cage were recorded at the same time, but in separate rooms with no other rats present. One rat of each cage was isolated in its home cage, its cage mate in a clean Makrolon IV cage with fresh sawdust. If there was a third cage mate, it was temporarily housed in a separate cage in the storage room. Recording started immediately after both rats had been placed in the recording rooms, and lasted 6 min. All recordings were performed between 9:30 and 14:00 h. The order of recording and the assignment of rats to treatment were done according to an a-priori assessed semi-random schedule.

For ultrasonic recording, two UltraSoundGate Condenser Microphones CM16 (Avisoft Bioacoustics) were used. They were placed 30 cm above the centre of the cage floor and were connected to the Avisoft devices as described under Experiment A. Ultrasonic calls were recorded with a sampling rate of 166,666 Hz in 16 bit format. Acoustical analysis was performed as in Experiment A, and the number of 50-kHz calls was determined. On the basis of their shape, calls were classified into flat and frequency modulated ones as described above.

3.1.3. Statistical analysis

Since calling in the Long-Evans strain did not differ between enriched and standard housed rats ($t_6>1.34$, $p>.05$), their results were collapsed across housing conditions. In order to assess whether rats in the home cage called more or less than their cage mates in a novel cage, the number of 50-kHz calls of both were compared using a GLM for repeated measures (recording in home versus novel cage) with strain as the between-subject factor. A GLM for repeated measurements was also used to compare the ratio between flat and frequency modulated calls. To assure normal distribution and homogeneity of variance of the data, the log transformed values plus 1 were used ([59], p. 378).

3.2. Results

As in Experiment A, rats emitted 50-kHz calls in a novel clean cage when separated from their cage mates (see Fig. 4). In line with the prediction, it was found that rats, which stayed alone in the home cage, also emitted 50-kHz calls. Surprisingly, they called even more than the rats in the novel cages (factor test condition: $F_{1,13}=15.17$, $p=.002$). This effect was found in both strains (no interaction between strain and test condition: $F_{1,13}=4.08$, $p=.062$), although Wistar rats called more than Long-Evans rats (factor strain: $F_{1,11}=16.67$, $p=.001$). As in Experiment A, calls emitted were mostly flat (74.82±3.86%), and this was found irrespective of whether rats were tested alone

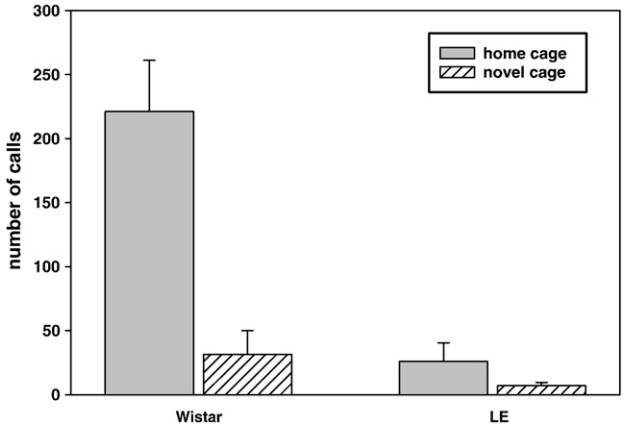

Fig. 4. The number of 50-kHz calls emitted either by Wistar (left) or Long-Evans (LE, right) rats in the home cage (grey bar) or in a novel cage (dashed bar). Data reflect means±SEM.

in the home cage (70.83±4.74%) or in a novel cage (77.53±5.75%, factor test condition: $F_{1,11}=.64$, $p=.440$). However, Long-Evans rats emitted more flat calls (82.58±2.44%) than Wistar rats (65.77±2.64%; strain: $F_{1,11}=20.221$, $p=.001$; no interaction between strain and test condition: $F_{1,11}=2.53$, $p=.140$).

4. General discussion

The present studies show that male adult rats have rather high rates of 50-kHz calling during transient isolation in a housing cage with bedding containing rat scents from the home cage. Rats even showed high numbers of 50-kHz calls when tested in clean cages containing fresh bedding. During open field and elevated plus maze testing, 50-kHz calls were also recorded, but at very low rates. Importantly, 50-kHz calling was strongly affected by prior experience, since calling in the housing cage test barely occurred in rats that had undergone appetitive discrimination training. Experiment B gave evidence that this effect of experience could be explained by the time since separation from the cage mate. It was found that separation also induced 50-kHz calling in the cage mates that remained alone in the home cage. Finally, evidence was provided that 50-kHz calling is dependent on individual factors, since call production varied substantially between rats, but was highly stable over days and was related to other behavioral measures, as obtained in the open field.

4.1. 50-kHz calling occurred in contexts that are not necessarily appetitive to rats

A bulk of evidence from ethological, pharmacological and brain stimulation studies, shows that 50-kHz calling is positively related to reward and negatively related to aversion, as indicated by place preference, self-administration, and instrumental approach [2,18,27]. The present findings, however, indicate that high rates of 50-kHz calling can also occur in contexts that are not necessarily pleasurable and appetitive to rats. Although the highest rate of 50-kHz calling found in the present study (76.9 calls/min by the naïve rats during the first minute of day 1) is not as high as the highest rates found during tickling (about 200 calls/min, [18], Fig. 2), it is much higher than the highest rate reported for a similar context of scented novel cages (about 17 calls/min, [32], p. 75). Rats in unscented cages on day 2 also had higher calling rates (30.5 calls/min during the first minute). The low call rates reported by Brudzynski and Pniak [32], but also the low rates (about 6 calls/min) found by Schwarting et al. ([19], Fig. 2) in test cages with fresh bedding, could probably be due to the fact that these rats had no cage mate. In fact, social experience [43] and housing conditions, whether singly or in groups [27], are known to modulate calling behavior.

The current results now clearly consolidate earlier suggestions by Schwarting et al. [19] that olfactory cues from other rats are not a prerequisite for substantial calling. The alternative explanation that calling in unsoiled cages on day 2 is solely based on Pavlovian association of scents and context on day 1 is implausible, since animals also emitted 50-kHz calls in Experiment B without such association. Nevertheless, in line with Brudzynski and Pniak [32], calling rates in the unscented cages were lower, albeit only in the first minute. This result, however, is flawed by the fact that the two subgroups had shown unexpected a-priori differences on the day before, where rats to be tested in a soiled cage had higher call rates.

When compared to the housing cage tests, 50-kHz call rates in the open field and the plus maze were rather low in the present study, but this could be explained by the possible aversiveness of these tests [15]. Accordingly, anxiety-related behavior was observed, namely avoidance of the centre and open arms. Nevertheless, the present data, especially the calls during plus maze testing under bright white light, add to other examples of 50-kHz calling in potentially aversive situations, like anticipation of an attacking opponent [36,37], aggressive encounters [8,13,33–41], and states of drug withdrawal [43].

4.2. 50-kHz calling was strongly affected by prior experience

In Experiment A, it was also found that the emission of 50-kHz ultrasonic calls was substantially affected by preceding experience. Rats which had been repeatedly handled and tested in other environments not only emitted fewer 50-kHz calls than the naïve rats, but their calls were also shorter, lower in amplitude and frequency, and were less frequency modulated. When tested in the open field and plus maze after termination of the appetitive training one week later, these rats vocalized more than naïve rats. These results show the importance of preceding experiences, which seem to have distinct and even inversed short- and long-term consequences on 50-kHz calling. Since treatment of the 'experienced' rats differed in a number of ways from that of naïve rats, which included extra handling and repeated exposures to T-mazes with food rewards, the effects cannot be attributed to a specific factor of experience, and will require specific testing in future studies. Furthermore, the possibility that the difference in calling rates between the experimental groups was related to some unknown reward aspect cannot be excluded, since the appetitive value of the housing cage test was not specifically tested.

4.3. The social function of 50-kHz calling

Several hypotheses have been proposed regarding the possible function of 50-kHz calls, like echo-location and others (for some early hypotheses see [60]). Currently, two major hypotheses regarding 50-kHz calls exist, which are not mutually exclusive: 1) calling as an affective expression [2], and 2) calling as a social signal [32,35,60]. The present findings are more in line with the social hypothesis that 50-kHz calls serve to maintain or (re-)establish social contact ("to call for somebody"). The occurrence of 50-kHz calls in non-rewarding situations and the high but decreasing rates of calling in the housing cage test can be explained by the

assumption that rats call for their cage mates. It also would explain why trained rats vocalized much less — they had been taken out of their home cage earlier and might have already reduced vocalization during that period. The corollary prediction that rats remaining alone in the home cage would also vocalize after separation was confirmed in Experiment B. Moreover, these rats vocalized even more than their cage mates which were placed in a novel cage. Therefore, the 50-kHz calls emitted in Experiment A cannot simply be attributed to features of the novel cage, e.g. scents of other rats or joy of exploration. Remarkably, similar effects were obtained in Long-Evans rats and Wistar rats, although call rates and inter-individual variability were higher in Wistar rats. This is not necessarily due to strain differences, since the two groups also differed in age and prior experience. Either way, these results show that it is important to take into account factors like strain, age and experience.

:The social hypothesis is not necessarily in contrast with the affective hypothesis, since 50-kHz calls can serve several purposes. Firstly, communicating a positive affective state by 50-kHz calls may have a function in inducing playfulness [18,61], i.e. "come on and play" [17]. Likewise, it has been suggested that rats may call to signal that they are "approaching in a friendly manner" [32]. Indeed, rats that enter an environment where social contact can be expected emit 50-kHz calls [20,32,36,37]. Secondly, vocalizations can reveal several types of information at the same time, e.g. the species, sex, condition, intention, location and identity of the sender, and especially 50-kHz calls potentially harbor many possible ways to convey subtle types of information. In fact, 50-kHz calls are known for their intra-individual variation, and accumulating evidence indicates that there are actually several call subtypes [8,21–23]. Recently, Burgdorf and Panksepp [45], who divided 50-kHz calls into flat and frequency modulated ones, showed that tickle responders primarily emitted frequency modulated calls. Further evidence that only the frequency modulated variety reflects a positive affective state, is provided by the fact that playback of frequency modulated calls is self-administered, whereas playback of flat calls is not [30]. Finally, Burgdorf et al. [62] have shown that the disruption of the mesolimbic dopamine system either by lesions or pharmacological blockade specifically reduce frequency modulated calls. Thus, it seems to be likely that the conflicting findings of 50-kHz calls in rewarding and non-rewarding or even mildly aversive contexts can be solved by using the distinction between flat and frequency modulated calls. Indeed, during natural behaviors that are clearly rewarding, i.e. rough-and-tumble play and mating, about 90% of the calls were frequency modulated, whereas during aggressive behavior the majority of the 50-kHz calls (about 65%) were flat [30]. Since the proportion of call subtypes in the present study more closely match the findings during aggressive behavior, it seems plausible that flat 50-kHz calls have a more social-coordinating function. Such a distinction may find parallels in humans where the unfelt social smile is a communicative gesture, and the Duchenne smile one that is affectively veridical [63]. At the very least, the present data show that social separation may be a useful and easy method for eliciting this barely understood call variant for further study.

4.4. Individuality

As found in several other studies (e.g. [15,16,19,29,45]), huge inter-individual variability in call emission was observed. Part of this variability can be explained by consistent individual differences, as not only call number and total calling time, but also additional acoustic parameters, like mean call duration, peak frequency and amplitude were significantly correlated between housing cage tests 1 and 3. This consistent inter-individual variety may therefore reflect dispositions or traits of the subject [19]. Moreover, individual differences in call production were related to differences in overt behavior, i.e. locomotion in an open field as tested three weeks later. Individual differences in undrugged locomotion in an unfamiliar open field are thought to gauge the expression of a sensation-seeking trait in the rat [49], which is related to striatal dopamine [50,51], which, in turn, is related to the production of 50-kHz calls [26,28,62]. In contrast to open field behavior, plus maze behavior was not related to ultrasonic calling (but see [19]), possibly due to a ceiling effect based on a rather anxiogenic testing procedure, which may have masked individual differences.

5. Conclusion

The present finding of substantial 50-kHz calling rates in non-rewarding situations shows that the occurrence of 50-kHz calls is apparently not restricted to appetitive contexts. The finding that separation from the cage mates also induced 50-kHz calling in rats remaining in the home cage, suggests that the calls are also used to (re)establish or maintain contact. This also holds for the reduced calling in the experienced rats which were separated for a longer period before being tested. These data show that (social) experience is an important factor to consider when interpreting vocalization data. The finding of the relatively high proportion of flat 50-kHz indicates that this call variant may have primarily a social-coordinating function.

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Studie IV

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Ultrasonic Communication in Rats: Can Playback of 50-kHz Calls Induce Approach Behavior?

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Rats emit distinct types of ultrasonic vocalizations, which differ depending on age, the subject's current state and environmental factors. Since it was shown that 50-kHz calls can serve as indices of the animal's positive subjective state, they have received increasing experimental attention, and have successfully been used to study neurobiological mechanisms of positive affect. However, it is likely that such calls do not only reflect a positive affective state, but that they also serve a communicative purpose. Actually, rats emit the highest rates of 50-kHz calls typically during social interactions, like reproductive behavior, juvenile play and tickling. Furthermore, it was recently shown that rats emit 50-kHz calls after separation from conspecifics. The aim of the present study was to test the communicative value of such 50-kHz calls. In a first experiment, conducted in juvenile rats situated singly on a radial maze apparatus, we showed that 50-kHz calls can induce behavioral activation and approach responses, which were selective to 50-kHz signals, since presentation of 22-kHz calls, considered to be aversive or threat signals, led to behavioral inhibition. In two other experiments, we used either natural 50-kHz calls, which had been previously recorded from other rats, or artificial sine wave stimuli, which were identical to these calls with respect to peak frequency, call length and temporal appearance. These signals were presented to either juvenile (Exp. 2) or adult (Exp. 3) male rats. Our data clearly show that 50-kHz signals can induce approach behavior, an effect, which was more pronounced in juvenile rats and which was not selective to natural calls, especially in adult rats. The recipient rats also emitted some 50-kHz calls in response to call presentation, but this effect was observed only in adult subjects. Together, our data show that 50-kHz calls can serve communicative purposes, namely as a social signal, which increases the likelihood of approach in the recipient conspecific.

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INTRODUCTION

Rats emit distinct types of ultrasonic vocalizations (USV), which differ depending on age, the subject's current state and environmental factors [1–3]. Rat pups typically exhibit USV in response to isolation from mother and litter [4]. Juvenile and adult rats, on the other hand, produce two different types of USV, which have been classified primarily on the basis of their sound frequency as low and high frequency vocalizations.

Low frequency vocalizations, often termed 22-kHz calls, are emitted when rats are exposed to predators [5], foot-shocks [6–10], during inter-male aggression [11,12], drug withdrawal [13,14], handling [15], and social isolation [16]. Remarkably, anxiolytic drugs can reduce such vocalizations [17–19]. Functionally, it was assumed that 22-kHz calls reflect a negative affective state akin anxiety and sadness [8,9], and that they serve as alarm cries [5].

Conversely, high-frequency vocalizations, often termed 50-kHz calls, occur during or in anticipation of juvenile rough-and-tumble play [19,20], mating [21–28], food consumption [29], electrical self-stimulation of the brain [29,30], and addictive drugs [31–35]. Furthermore, rats also emit such calls when tickled by a skilled experimenter in a playful way [36–40], and rates of 50-kHz calls were found to be positively correlated with the rewarding value of tickle stimulation as measured by instrumental approach behavior [36,37,39]. Conversely, aversive stimuli including bright light [20,37], predatory odors [37], the presence of foot shock cues [29] and drugs with aversive properties decrease levels of 50-kHz calls [41]. Based on such evidence, Panksepp and Burgdorf [40] suggested that 50-kHz calls might provide an archaic form of human laughter ("rat laughter"), which might serve as an index of the animal's subjective state [2]. Thereby, 50-kHz calls might provide a new and unique measure for analyzing natural reward circuits in the brain [29,30,42].

Recently, however, it was shown that 50-kHz calls can also occur in situations that are not necessarily pleasurable or even mildly aversive to rats. Thus, it was found that 50-kHz calls were emitted during short social isolation in the animal's own, or in a new soiled or fresh housing cage, irrespective of whether the animal's motivational status was high or low, i.e. irrespective of whether the animal was food-deprived or fed ad libitum [40,43]. Also, during testing in an open field and an elevated plus maze 50-kHz calling was observed [43]. These findings are in line with observations of 50-kHz calls in various experimental controls, like naïve rats that were placed into a test arena containing fresh bedding [24,44], or saline-injected rats in drug studies [33–35,41]. Remarkably, the propensity to call differed dependent on the time-point of the last social contact, i.e. rats emitted 50-kHz calls primarily initially after separation from the cage mate [43]. Finally, it was found that not only the animal, which was isolated

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in a new housing cage emitted 50-kHz calls, but also the cage mate that remained alone in the home cage after the removal of the test rat [43]. These findings corroborated the idea that 50-kHz calls serve for communicative purposes, e.g. to (re)establish or keep contact.

A social function of rat USV was already confirmed successfully by performing playback studies in pups [45–47]. In adult rats, it was shown that the presentation of natural 22-kHz calls or 20-kHz sine wave tones can activate the fight/flight/freeze system [48–53]. However, little is known about the effects of 50-kHz calls on the behavior of the receiver. Schleidt [54] found that diverse artificial ultrasonic stimuli elicit Preyer's reflex, i.e. twitches of the auricles, in rats, and Thomas et al. [55] observed a suppression of instrumental bar pressing and bradycardia when artificial 50-kHz tones were presented. Apart from these early studies, responses to playback of high-frequency ultrasonic stimuli have been studied primarily within the sexual context. Here, changes in approach behavior [56,57], proceptive behavior [22,25,27] and ultrasonic calling were observed [58]. Finally, two recent studies in non-sexual contexts obtained incongruent results. Burgdorf et al. [32] found that rats show instrumental behavior to receive playback of 50-kHz calls, whereas Endres et al. [59] did not find overt behavioral effects of 50-kHz playback.

The aim of the present study was to test the communicative value of 50-kHz calls by measuring overt and calling behavior during playback of such calls. As a testing environment, we used an unbaited radial-arm maze, since this apparatus had proven its usefulness in a previous experiment, where we had tested the behavioral effects of presenting pup 40-kHz calls to rat dams [47]. Here, it was hypothesized that presentations of 50-kHz calls induce locomotor activity and ultrasonic calling, whereas 22-kHz calls induce locomotor inhibition and a reduction in ultrasonic calling (Exp. 1). Furthermore, it was hypothesized that the 50-kHz call induced activation is stimulus-directed, i.e. that animals will approach the source of 50-kHz calls while calling themselves. Also, we assumed that the behavioral response is dependent on subject- and call-related features. Regarding subjects, we used juvenile (Exp. 1 & 2) and adult rats (Exp. 3), expecting stronger behavioral responses in juvenile rats, where 50-kHz calls occur in great numbers [37]. To test the effect of call features, natural 50-kHz calls and artificial sine wave tones (i.e. ''calls'' without amplitude and frequency modulation) were used (Exp. 2 & 3). In accordance to a bulk of evidence showing that primarily frequency modulated 50-kHz calls are linked to a positive affective state [30,32,42], it was expected that they can induce approach behavior. However, it was expected that flat 50-kHz signals might also induce approach behavior, since it was shown that flat calls are predominantly emitted after separation from the cage mate, suggesting that this call serves as a contact call [40,43].

MATERIALS AND METHODS

Animals and housing

In total, 68 male Wistar rats (HsdCpb:WU, Harlan-Winkelmann, Borchen, Germany) served as subjects. In Exp. 1, 12 juvenile male rats were used, weighing 66.7±2.5 g (range: 52.5–76.5 g; about 25 days of age) on the test day. Twenty juvenile male rats were used in Exp. 2, weighing 80.9±1.5 g (range: 66.0–91.0 g; about 27 days of age) on the test day. Finally, 36 adult male rats were used in Exp. 3, weighing 320.5±6.3 g (range: 273.0–422.0 g; about 12 weeks of age) on the test day. All animals were naïve, except for animals of Exp. 2, which were separated from mother and litter two times for 10 min on postnatal day 11. Animals were housed in groups of 5 (Exp. 2) or 6 (Exp. 1 & 3) on Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland) in a Macrolon type IV cage (size: 378×217×180 mm, plus high stainless steel covers). Lab chow (Altromin, Lage, Germany) and water (0.0004% HCl-solution) were available ad libitum. Animals were housed in an animal room with a 12:12 h light/dark cycle (lights on 7–19 h) where the environmental temperature was maintained between 20–25° Celsius. Prior to testing, all animals were handled for 3 days in a standardized way (5 min each day).

Experimental setting

Testing was performed on a radial maze of gray plastic with 8 arms (9.8×40.5 cm) extending radially from a central platform (diameter: 24 cm), which was elevated 52 cm above the floor (for details see: [60]). Acoustic stimuli were presented through an ultrasonic speaker (ScanSpeak, Avisoft Bioacoustics, Berlin, Germany) using an external sound card with a sampling rate of 192 kHz (Fire Wire Audio Capture FA-101, Edirol, London, UK) and a portable ultrasonic power amplifier with a frequency range of 1–125 kHz (Avisoft Bioacoustics). The loudspeaker had a frequency range of 1–120 kHz with a relatively flat frequency response (± 12 dB) between 15–80 kHz. It was placed 20 cm away from the end of one arm at a height of 52 cm above the floor. Testing was performed under red light (approximately 11 lux in the center of the maze and between 9 and 12 lux in the arms) in a testing room with no other rats present.

All behavioral tests were conducted between 9–17 h. Prior to each test, behavioral equipment was cleaned using a 0.1 % acetic acid solution followed by drying.

Acoustic stimuli

The following four acoustic stimuli were used: 50-kHz calls, 50-kHz sine wave tones, 22-kHz calls, and background noise (see Fig. 1). All stimuli were presented for 1 min with a sampling rate of 192 kHz in 16 bit format. Calls and tones were presented at about 69 dB (measured from a distance of 40 cm), and noise was presented with about 50 dB, which corresponds to the background noise during playback of the other stimuli.

50-kHz calls

Throughout playback, 221 natural 50-kHz calls (total calling time: 15.3 s) were presented. The presentation was composed of a sequence of 3.5 s, which was repeated for 1 min, i.e. 17 times, to assure the presentation of a high number of frequency-modulated calls within a relatively short period of time. Each sequence contained 13 calls (total calling time: 0.90 s). Out of these, 10 were frequency-modulated and 3 were flat, and had the following features: call duration 0.07±0.01 s (mean±SEM); peak frequency: 61.24±1.75 kHz; bandwidth: 4.63±1.21 kHz; frequency modulation: 31.68±4.62 kHz. These calls had been recorded from a male Wistar rat during exploration of a cage containing scents from a cage mate (for setting and recording see: [43]).

50-kHz tones

50-kHz sine wave tones were generated with the computer software SASLab Pro (version 4.2, Avisoft Bioacoustics) by replacing all calls through sine wave tones. In detail, each given call was replaced by a sine wave tone with identical duration, frequency, amplitude, etc. Thus, the signal had the same temporal patterning and was identical to the 50-kHz call signal with respect to all call features, apart from the fact that the tones were not amplitude and frequency modulated as the natural 50-kHz calls.

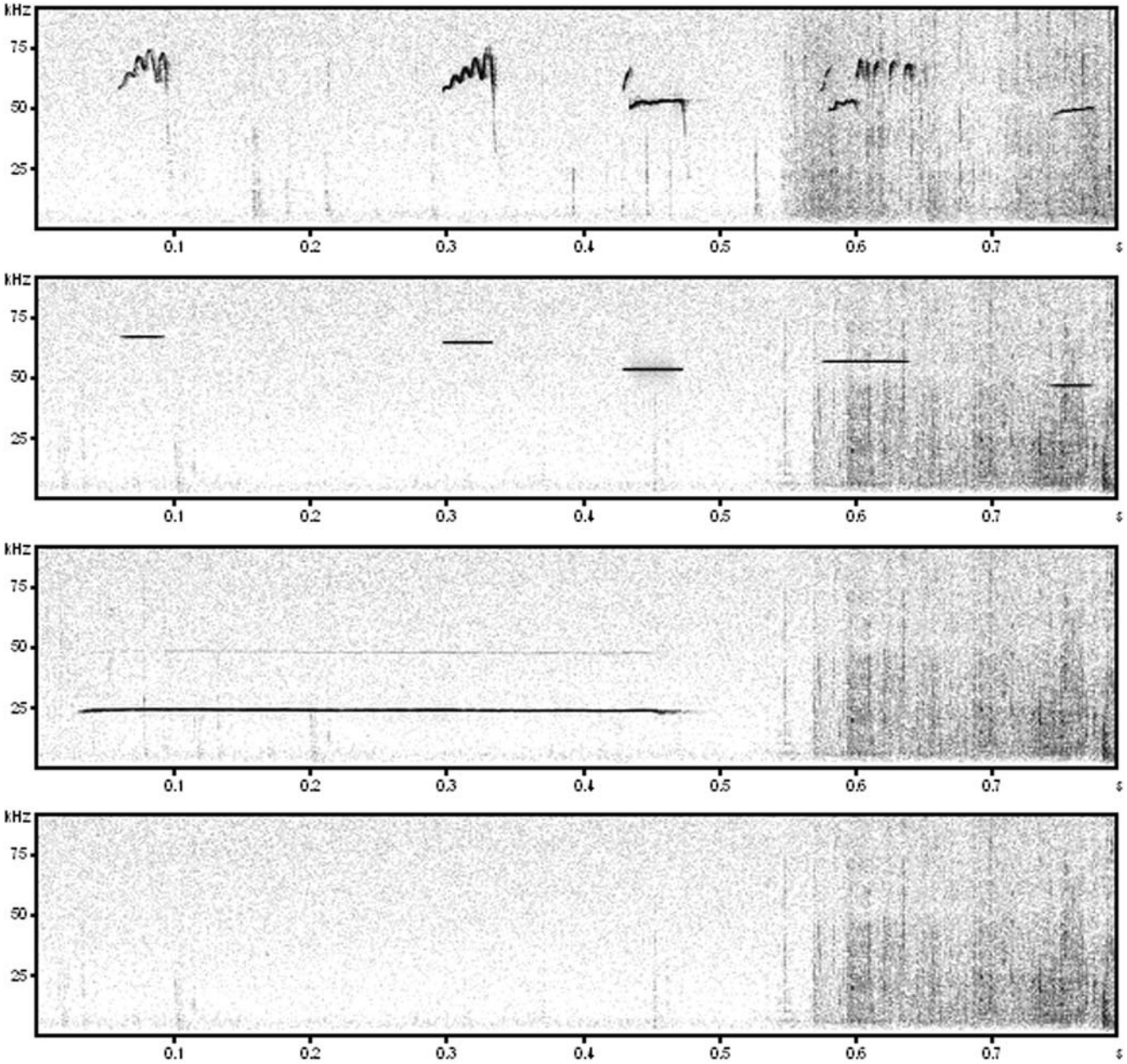

Figure 1. Exemplary spectrograms of the four types of acoustic stimuli presented, namely (from top to down): natural 50-kHz calls, artificial 50-kHz sine wave tones, natural 22-kHz calls, and background noise.
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22-kHz calls

Throughout playback, 29 natural 22-kHz calls (total calling time: 34.25 s) were presented. These calls had the following acoustic parameters: call duration 1.18±0.06 s; peak frequency: 23.61± 0.07 kHz; bandwidth: 1.37±0.05 kHz; frequency modulation: 1.90±0.09 kHz. Their presentation was not composed of a repeated sequence, since in case of the long 22-kHz calls potential information, which is contained in temporal patterning is likely lost through sequencing. These calls had been recorded from a male Wistar rat after applications of foot-shocks (for setting and recording see: [10]).

Noise

Since all three acoustic stimuli presented contained background noise, i.e. sounds, which occur when a rat is exploring an arena with bedding, background noise without calls or tones was presented to control for its possible effects.

Experimental procedure

A given animal was placed onto the central platform of the radial maze, facing the arm opposite to the loudspeaker. After an initial phase of 15 min where no acoustic stimuli were presented (termed habituation), the rat was exposed to three presentations of acoustic

stimuli for 1 min, each followed by an inter-stimulus-interval of 10 min.

Between sub-groups of subjects, different orders of stimulation presentation were used to account for the possible impact of sequence effects. In Exp. 1, background noise, 22-kHz calls and 50-kHz calls were used as acoustic stimuli. They were presented in the following orders: a) background noise, b) 22-kHz calls, c) 50-kHz calls (n = 6 rats), or a) background noise, b) 50-kHz calls, c) 22-kHz calls (n = 6). In Exp. 2 and 3, where background noise, 50-kHz sine wave tones and 50-kHz calls were tested used, they were presented either in the order a) background noise, b) 50-kHz sine wave tones, c) 50-kHz calls (Exp. 2: n = 6; Exp. 3: n = 12), or a) background noise, b) 50-kHz calls, c) 50-kHz sine wave tones (Exp. 2: n = 6; Exp. 3: n = 12), or a) 50-kHz calls, b) 50-kHz sine wave tones, c) background noise (Exp. 3: n = 12), or a) 50-kHz calls, background noise, 50-kHz sine wave tones (Exp. 2: n = 7). One animal was excluded from analysis of Exp. 2 due to incorrect presentation of acoustic stimuli.

We abstained from depicting the order of stimulus presentation in detail, since it had no major qualitative effects on the patterns of result, i.e. behavioral responses towards 22-kHz calls and 50-kHz calls were similar over all positions (Mann-Whitney-U-test for Exp. 1 or Kruskal-Wallis-test for Exp. 2 & 3: all p-values >.100).

Recording and analysis of animal activity

Behavior was monitored by a video camera (Panasonic WV-BP 330/GE, Hamburg, Germany) from about 150 cm above the maze, which fed into DVD recorder (DVR-3100 S, Pioneer, Willich, Germany).

Behavioral analysis was performed in two ways. A trained observer scored the videos for the time spent on the three arms proximal to or distal from the ultrasonic loudspeaker. Furthermore, the total distance travelled (cm), and the number of arm entries into the three proximal or distal arms, were analyzed using an automated video tracking system (Ethovision, Noldus, Wageningen, The Netherlands). For the automated analysis, input filters were activated to avoid an over-estimation of locomotor activity due to head-movements. In more detail, a minimal distance moved of 8 cm was used for the total distance travelled, whereas a minimal distance moved of 3 cm was used for the arm entries.

Recording and analysis of ultrasonic vocalization

Playback of acoustic stimuli and potential ultrasonic calls uttered by the rat under testing were monitored by two UltraSoundGate Condenser Microphones (CM 16; Avisoft Bioacoustics) placed 20 cm away from the maze at a height of 55 cm above the floor. One out of these two was placed next to the loudspeaker, i.e. in front of the three proximal arms, whereas the other one was placed vis-à-vis in front of the three distal arms. These microphones were sensitive to frequencies of 15-180 kHz with a flat frequency response (± 6 dB) between 25–140 kHz, and were connected via an Avisoft UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics), and were recorded with a sampling rate of 214,285 Hz in16 bit format.

For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100 % frame, Hamming window and 75 % time window overlap). Correspondingly, the spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. The numbers of 22-kHz calls and 50-kHz calls were counted by experienced observers.

Statistical analysis

Non-parametric statistics were used, since several data sets were not normally distributed as indicated by the Shapiro-Wilk-test. In more detail, the Friedman-test for repeated measurements was calculated to test whether overt or calling behavior is affected by presentation of acoustic stimuli. When appropriate, the Wilcoxon-test was used subsequently to determine whether overt or calling behavior during presentation of a given acoustic stimulus differ in comparison to other acoustic stimuli, or in comparison to phases without presentations of acoustic stimuli. For the last purpose, overt and calling behavior shown in the three min preceding stimulus application was averaged to eliminate habituation effects. Furthermore, the Wilcoxon-test was used to compare the entries into or the time spent on proximal or distal arms of the radial-maze during a given test period. Finally, Spearman correlation coefficients were calculated to test whether individual responses to different acoustic stimuli were stable and whether overt and calling behaviors were related to each other. The exact p-values of 2-tailed testing were taken as measures of effect.

RESULTS

Experiment 1 – juvenile rats

This initial experiment was performed to test whether presentation of ultrasonic calls is effective to modify behavior in juvenile rats. Here, we used 22-kHz calls, for which we expected behavioral inhibition, and natural 50-kHz calls, for which we expected activation and orientation towards the source of stimulation.

Locomotor activity

Locomotor activity of juvenile rats was affected by presentations of acoustic stimuli (see Fig. 2), since the distance travelled was dependent on a) whether acoustic stimuli were presented or not and b) which type of stimulus was presented. In detail, natural 50-kHz calls caused an increase in the distance travelled in comparison to test periods without presentations ($Z = -2.353$, $p = .016$), or to presentation of noise ($Z = -2.934$, $p = .001$). In contrast, locomotor activity was reduced when natural 22-kHz calls were presented, indicated by a decrease when compared versus natural 50-kHz calls ($Z = -2.746$, $p = .003$), and a trend for a decrease in comparison to test periods without presentations ($Z = -1.955$, $p = .055$), but not in comparison to presentation of noise ($Z = -.415$, $p = .734$). Finally, no difference in locomotor activity was found between test periods without presentations and background noise ($Z = -1.070$, $p = .322$).

Stimulus-directed locomotor activity

As expected, only natural 50-kHz calls, but not natural 22-kHz calls, induced approach behavior (see Movie S1). Thus, during presentations of 50-kHz calls animals entered the three proximal arms in front of the loudspeaker more often than the three distal ones ($Z = -2.456$, $p = .016$) and spent more time in the former ($Z = -3.059$, $p < .001$). No preference was observed during playback of noise or natural 22-kHz calls (all p-values >.100). Remarkably, approach behavior during playback of 50-kHz calls was evident despite the fact that the animals showed an a-priori preference for the distal arms, indicated by more entries into distal arms than in proximal ones and the fact that animals spent more time in the distal arms than proximal ones during habituation ($Z = -2.185$, $p = .026$ and $Z = -2.510$, $p = .009$, respectively) and after cessation of noise ($Z = -1.720$, $p = .084$ and $Z = -2.134$, $p = .032$, respectively). After playback of 22-kHz calls, no preference was found (all p-values >.100), whereas animals

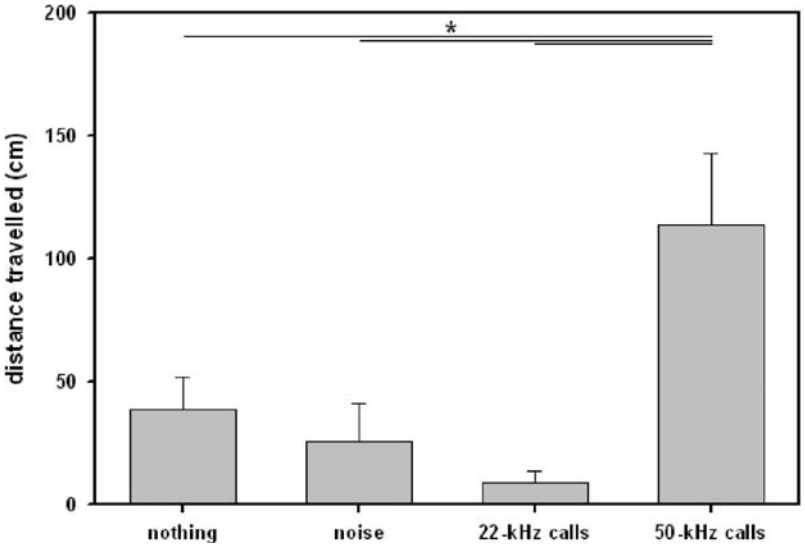

Figure 2. Locomotor activity of juvenile rats in Exp. 1. Bars depict the distance travelled during test phases without acoustic presentation (nothing), presentation of noise (noise), artificial 50-kHz sine wave tones (50-kHz tones), and natural 50-kHz calls (50-kHz calls). Values reflect means±SEM per minute. Animals of all stimulus orders were collapsed, i.e. n = 12. Comparisons with $p<.05$ are marked with asterisks: *.
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tended to stay longer in proximal arms than in distal ones after presentation of 50-kHz calls ($Z = -1.805$, $p = .076$; arm entries: $Z = -1.660$, $p = .110$). When comparing the time spent on proximal arms during playback of 22-kHz calls and 50-kHz calls, it was found that animals spent more time on proximal arms during playback of 50-kHz calls ($Z = -2.589$, $p = .007$; see Fig. 3). This stimulus-dependent difference was also evident after cessation of acoustic stimuli ($Z = -2.040$, $p = .042$), indicating that 50-kHz calls can induce a sustained preference for the source of playback.

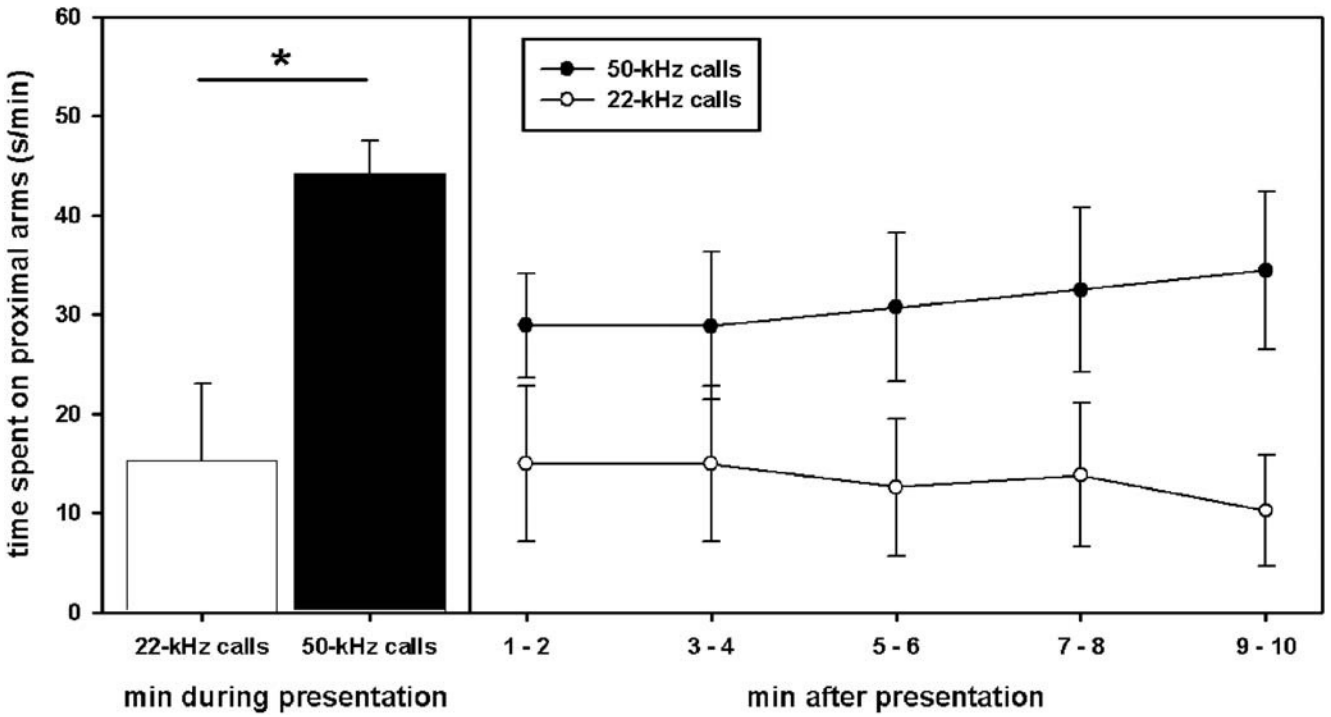

Figure 3. Stimulus-directed locomotor activity of juvenile rats in Exp. 1. The time spent on the proximal arms in front of the loudspeaker is given for playback of natural 22-kHz calls (white bar) and natural 50-kHz calls (black bar) is depicted on the left. On the right, the time spent on the proximal arms in front of the loudspeaker is given for the 10 min after cessation of playback of natural 22-kHz calls (open symbols) and natural 50-kHz calls (filled symbols). Values reflect means±SEM per minute. In both cases, animals of all stimulus orders were collapsed, i.e. n = 12. Comparisons with $p<.05$ are marked with asterisks: *.
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Ultrasonic calling

During testing, 7 out of 12 animals emitted some 50-kHz calls (1.75±0.65, i.e. 0.02±0.01 per min). However, none of them emitted 50-kHz calls during presentation of 50-kHz calls, or 22-kHz calls, and only one animal emitted a single call during presentation of noise, meaning that calls were predominantly emitted during inter-stimulus-intervals (not shown in detail).

22-kHz calls were not observed. However, calls with a similar shape and a long duration up to 900 ms, but an atypical high frequency, were found in one animal, which emitted 15 calls after cessation of presentations of 50-kHz calls (not shown in detail). Remarkably, it emitted also 50-kHz calls.

Experiment 2 – juvenile rats

Here, we again used juvenile subjects and tested whether behavioral activation and approach might not only be elicited by natural 50-kHz calls, but also by artificial 50-kHz sine wave tones which had the same temporal patterning and were identical to 50-kHz calls with respect to all call features, apart from the fact that the tones were not amplitude and frequency modulated.

Locomotor activity

In replication of Exp. 1, it was found that 50-kHz calls caused an increase in the distance travelled in comparison to test periods without presentations ($Z = -3.662$, $p < .001$), or to presentation of noise ($Z = -3.662$, $p < .001$; see Fig. 4). In contrast, playback of 50-kHz tones did not induce locomotor activation, and locomotor activity during presentation of 50-kHz tones was lower as during presentation of 50-kHz calls ($Z = -3.340$, $p < .001$; all other p-values $> .100$). Finally, no difference in locomotor activity was found between test periods without presentations and background noise ($Z = -1.046$, $p = .312$).

Stimulus-directed locomotor activity

Furthermore, it was found that locomotor activity was stimulus-directed during both, presentation of 50-kHz tones and natural 50-kHz calls (see Fig. 5), since the animals entered the three proximal arms in front of the loudspeaker more often than the distal ones (50-kHz tones: $Z = -2.012$, $p = .055$; 50-kHz calls: $Z = -3.572$, $p < .001$). Furthermore, they spent more time on the proximal arms than on the distal ones (50-kHz tones: $Z = -3.575$, $p < .001$; 50-kHz calls: $Z = -3.823$, $p < .001$). Such preferences were not observed during test periods without presentations, or during presentation of noise, except for a trend for a longer time spent on proximal arms relatively to distal ones after the cessation of presentation of 50-kHz calls ($Z = -1.811$, $p = .073$; all other p-values $> .100$).

Ultrasonic calling

During testing, 10 out of 19 animals emitted 50-kHz calls. However, call rates were very low (1.42±0.58, i.e. 0.03±0.01 per min), and none of them emitted 50-kHz calls during presentation of 50-kHz tones or 50-kHz calls. Solely 1 animal emitted 1 single call during presentation of noise, meaning that 50-kHz calls were predominantly emitted during ISIs (not shown in detail).

22-kHz calls were not observed. However, calls with a similar shape and a long duration up to 900 ms, but an atypical high frequency, were found in some few animals. Throughout the whole testing period, 3 out of 19 animals emitted them (1, 4 and 22 calls). Calls were primarily emitted during the presentations of 50-kHz tones or 50-kHz calls and after cessation of presentations (not shown in detail). Remarkably, 2 out of the 3 animals also emitted 50-kHz calls.

Experiment 3 – adult animals

In this final experiment, we used the same approach as in Exp.2, and asked whether 50-kHz calls or 50-kHz sine wave tones might also be effective when presented to adult rats.

Locomotor activity

As in juvenile rats, locomotor activity was dependent on on a) whether acoustic stimuli were presented or not and b) which type of stimulus was presented (see Fig. 6). In detail, 50-kHz calls caused an increase in the distance travelled in comparison to test periods without presentations ($Z = -.3833$, $p < .001$), or to noise

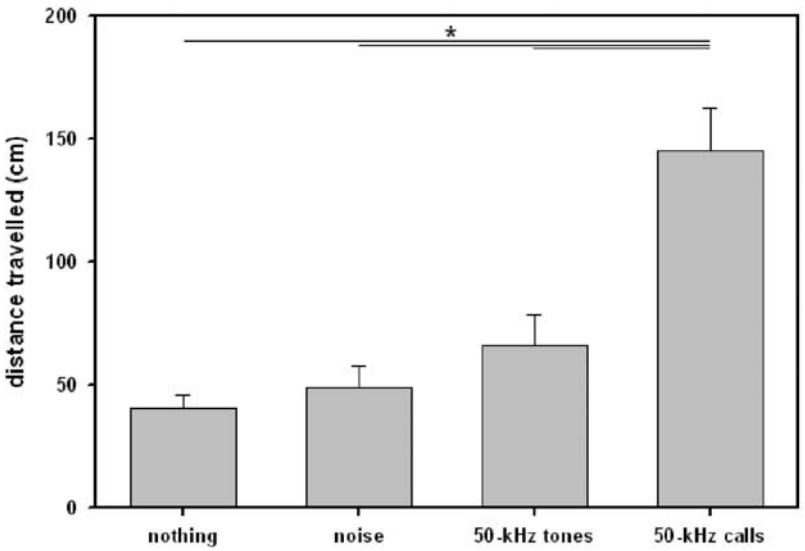

Figure 4. Locomotor activity of juvenile rats in Exp. 2. Bars depict the distance travelled during test phases without acoustic presentation (nothing), presentation of noise (noise), artificial 50-kHz sine wave tones (50-kHz tones), and natural 50-kHz calls (50-kHz calls). Values reflect means±SEM per minute. Animals of all stimulus orders were collapsed, i.e. n = 19. Comparisons with $p < .05$ are marked with asterisks: *.
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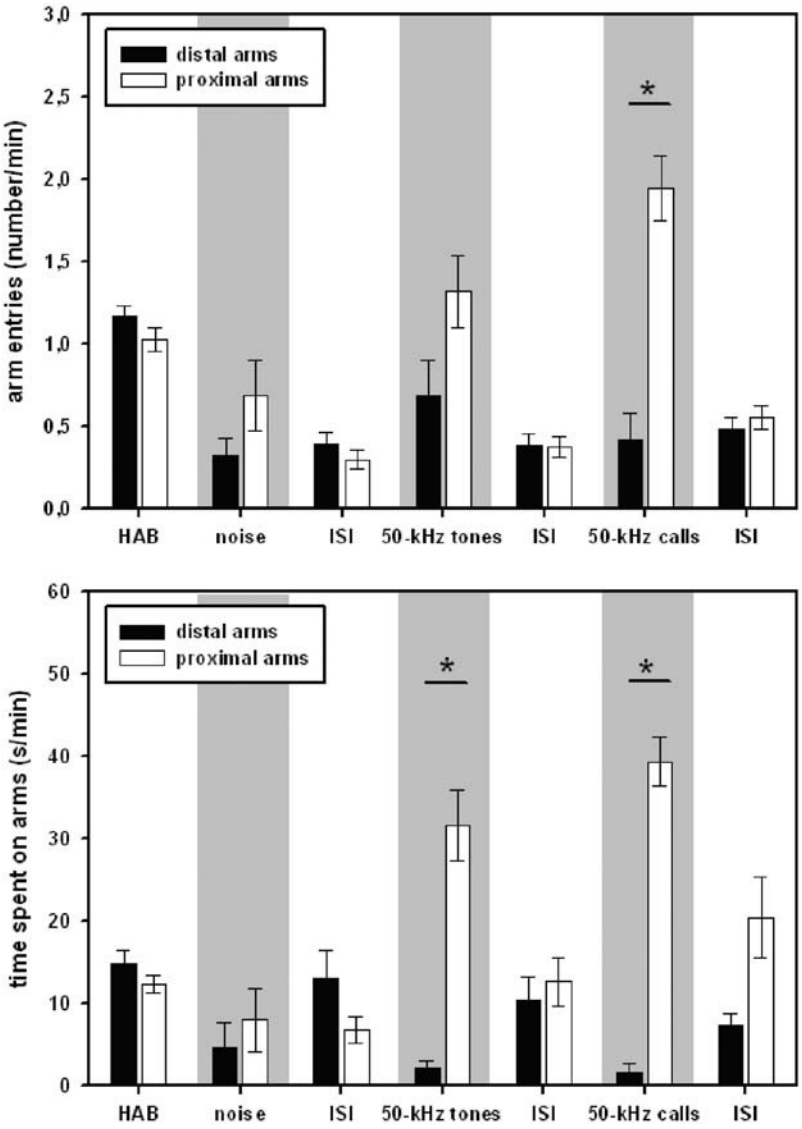

Figure 5. Stimulus-directed locomotor activity of juvenile rats in Exp. 2. The number of entries into the distal (black bars) or proximal (white bars) arms from the loudspeaker is given for habituation (HAB), inter-stimulus-intervals (ISI), and playback of acoustic stimuli, i.e. natural 50-kHz calls (50-kHz calls), artificial 50-kHz sine wave tones (50-kHz tones), and background noise (noise) in the upper figure. The time spent on the distal (black bars) or proximal (white bars) arms from the loudspeaker is given for habituation (HAB), inter-stimulus-intervals (ISI), and playback of acoustic stimuli, i.e. natural 50-kHz calls (50-kHz calls), artificial 50-kHz sine wave tones (50-kHz tones), and background noise (noise) in the bottom figure. Values reflect means±SEM per minute. In both cases, animals of all stimulus orders were collapsed, i.e. n = 19. Comparisons with $p<.05$ are marked with asterisks: *.
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($Z=-3.976$, $p<.001$). Furthermore, a similar increase in the distance travelled was observed when 50-kHz tones were presented (in comparison to periods without presentations: $Z=-3.620$, $p<.001$; in comparison to presentation of noise: $Z=-3.548$, $p<.001$). Remarkably, the distance travelled did not differ between presentations of 50-kHz tones and 50-kHz calls ($Z=-.131$, $p=.903$). Finally, no difference in locomotor activity was found between test periods without presentations and background noise ($Z=-1.456$, $p=.150$).

Stimulus-directed locomotor activity

Locomotor activity was stimulus-directed during presentations of 50-kHz tones and 50-kHz calls (see Fig. 7), since the animals entered the three proximal arms in front of the loudspeaker more often than the three distal ones ($Z=-4.110$, $p=.001$ and $Z=-3.155$, $p<.001$, respectively). Also, they spent more time on the proximal arms (50-kHz tones: $Z=-2.575$, $p=.008$; 50−kHz calls: $Z=-2.516$, $p=.010$). Such preferences were not observed during test periods without presentations or presentation of noise (all p-values $>.100$).

Ultrasonic calling

During testing, 26 out of 36 animals emitted 50-kHz calls (5.44±2.49, i.e. 0.11±0.05 per min). Out of these, 8 animals emitted 50-kHz calls during presentation of 50-kHz tones or 50-kHz calls, but none animal emitted 50-kHz calls during presentation of noise. Remarkably, 50-kHz calling was affected by presentations of acoustic stimuli (see Fig. 8). Call emission was higher during presentations of 50-kHz calls than during testing periods without presentation ($Z=-2.157$, $p=.047$) or presenta-

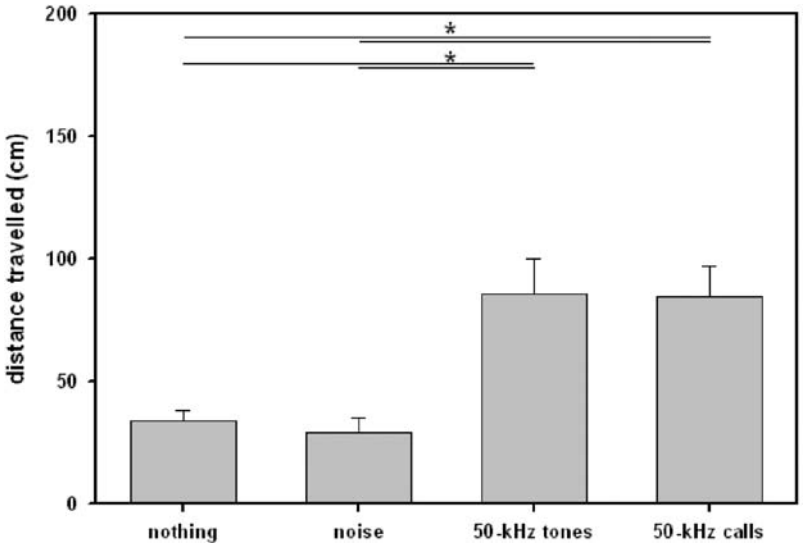

Figure 6. Locomotor activity of adult rats in Exp. 3. Bars depict the distance travelled during test phases without acoustic presentation (nothing), presentation of noise (noise), artificial 50-kHz sine wave tones (50-kHz tones), and natural 50-kHz calls (50-kHz calls). Values reflect means±SEM per minute. Animals of all stimulus orders were collapsed, i.e. n = 36. Comparisons with $p<.05$ are marked with asterisks: *.
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tion of noise ($Z = -2.410$, $p = .016$), whereas call emission during presentations of 50-kHz tones did not differ from any other test period (all p-values >.100), indicating that only playback of 50-kHz calls induced 50-kHz calling. Finally, no difference in calling behavior was found between test periods without presentations and background noise ($Z = -1.414$, $p = .500$).

Interestingly, 50-kHz calling was related to activity and approach behavior during presentations of 50-kHz tones and 50-kHz calls. In detail, during presentation of 50-kHz tones the number of 50-kHz calls emitted was positively correlated with the distance travelled (rho = .394, p = .017), the number of entries in proximal arms (rho = .404, p = .014) and the time spent there (rho = .346, p = .039), but not with the number of entries in distal arms (rho = .043, p = .803) and the time spent there (rho = .314, p = .062). During presentations of 50-kHz calls, the number of 50-kHz calls emitted by the subject under study was positively correlated with the distance travelled (rho = .345, p = .039), the number of entries in proximal arms (rho = .386, p = .020) and tended to correlate with the time spent there (rho = .299, p = .076), but no with the number of entries in distal arms (rho = .017, p = .922) and the time spent there (rho = −.147, p = .392) were observed. No correlations between 50-kHz calling and locomotor activity and the direction of locomotor activity were found during habituation (all p-values >.050).

22-kHz calls were very rarely observed. Throughout the whole testing period, only 2 out of 36 animals emitted them. One of them emitted 9 calls after cessation of the presentation of 50-kHz tones, the other one emitted 2 calls after cessation of the presentation of 50-kHz calls (not shown in detail). Remarkably, both animals emitted not only 22-kHz calls, but also 50-kHz calls. Actually, the first one displayed the highest number of 50-kHz calls throughout the whole testing period (90 calls), but also throughout the presentations 50-kHz tones (22 calls) and 50-kHz calls (32 calls).

DISCUSSION

Our results demonstrate for the first time that 50-kHz calls can induce approach behavior and ultrasonic calling in non-sexual contexts, whereas 22-kHz calls induced a reduction in locomotor activity.

Playback of 22-kHz calls induce behavioral inhibition

The present findings are in line with several previous ones, which have already shown that 22-kHz calls can activate the fight/flight/freeze system. Dependent on the strain of the receiver, 22-kHz calls can induce behavioral inhibition [48–51], or bursts of locomotor running and jumping, which are characteristic of defence behavior [49,50,52,53]. However, it has to be noted that studies using natural 22-kHz calls obtained only a moderate reduction of locomotor activity [48,51,59], which is in line with the relatively weak effects of 22-kHz calls found here. From these results, one should not conclude that 22-kHz calls do not provide important signals for the recipient; rather, one should assume that their salience depends on additional features like a given social context [5], or whether they are linked to critical experiences [59].

Playback of 50-kHz calls can induce activation and approach

Studies on the behavioral effects of 50-kHz calling using playback methods were predominantly conducted in the sexual context. Here, it was found that darting behavior and approaches toward the partner increased in frequency when the female was devocalized, but decreased when tape recorded female ultrasonic calls were presented [56,57]. With respect to male USV, it was shown that devocalization of male rats resulted in a reduction of female proceptive behavior [61], and playback of 50-kHz calls restored proceptive behavior in oestrus females [23,25,27].

The few studies, which were conducted in a non-sexual context, however, obtained very weak or no playback-induced effects on overt behavior. Thus, early studies using artificial ultrasonic stimuli observed Preyer's reflex [54], or a suppression of instrumental bar pressing and bradycardia [55], possibly reflecting an unspecific orienting response. Finally, a recent study by Endres et al. [59], did not observe any change in overt behavioral activity when natural 50-kHz calls were presented in comparison to other acoustic stimuli, like white noise or even 22-kHz calls. Therefore, the present study is the first one, which clearly shows that 50-kHz calls can affect overt and calling behavior in a non-sexual context.

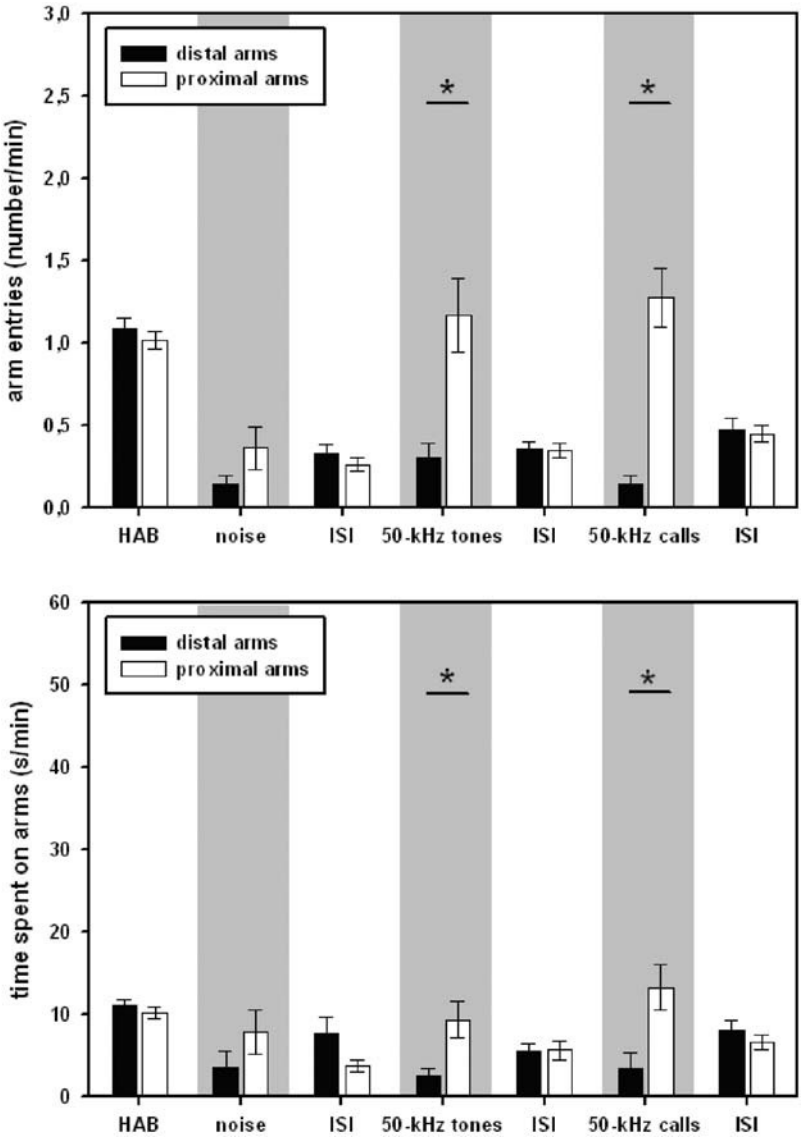

Figure 7. Stimulus-directed locomotor activity of adult rats in Exp. 3. The number of entries into the distal (black bars) or proximal (white bars) arms from the loudspeaker is given for habituation (HAB), inter-stimulus-intervals (ISI), and playback of acoustic stimuli, i.e. natural 50-kHz calls (50-kHz calls), artificial 50-kHz sine wave tones (50-kHz tones), and background noise (noise) in the upper figure. The time spent on the distal (black bars) or proximal (white bars) arms from the loudspeaker is given for habituation (HAB), inter-stimulus-intervals (ISI), and playback of acoustic stimuli, i.e. natural 50-kHz calls (50-kHz calls), artificial 50-kHz sine wave tones (50-kHz tones), and background noise (noise) in the bottom figure. Values reflect means±SEM per minute. In both cases, animals of all stimulus orders were collapsed, i.e. n = 36. Comparisons with $p<.05$ are marked with asterisks: *.
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In accordance to the hypothesis that 50-kHz calls serve communicative purposes [44,62,63], we found that animals increase locomotor activity and approach the source of the stimulus, resembling mothers when searching for their pups in response to isolation-induced pup calls [45,46,47].

Furthermore, we showed that playback of 50-kHz calls can elicit ultrasonic calling by the recipient subject, which is in line with findings by White et al. [58] showing that male 50-kHz calls can elevate female calling. Thus, the present findings clearly indicate that the communicative value of 50-kHz calls is not restricted to sexual interactions. Therefore, it can be concluded that differences between sexual and non-sexual contexts are not responsible for the conflicting findings. Possible reasons for the lack of evidence in previous studies might be due to the type of stimulus material and playback technology used in the early playback work [54,55], or the experimental setting used in the study of Endres et al. [59], who mounted their loudspeaker above the testing arena and not at the side, as done here. Possibly, 50-kHz signals coming from the horizontal axis might provide a more naturalistic signal for the recipient than calls coming from above.

Frequency modulation is not necessary for eliciting approach behavior

The fact that 50-kHz calls induced approach behavior clearly indicates that these calls were appetitive, which is in line with findings by Burgdorf et al. [32] who showed that rats show instrumental behavior to receive 50-kHz calls. There, frequency-modulated, but not flat 50-kHz calls were effective, whereas the present results demonstrate that 50-kHz signals with and without

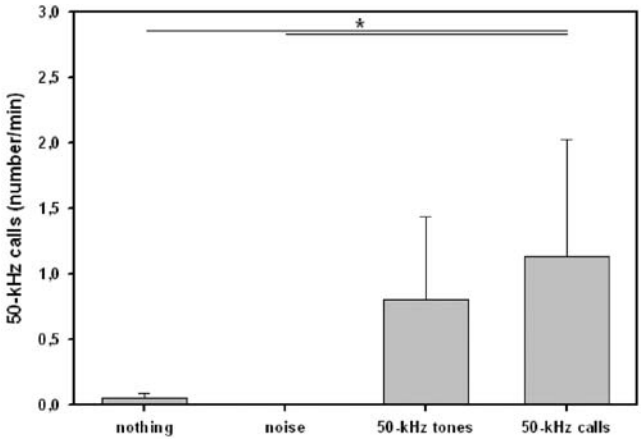

Figure 8. Ultrasonic calling of adult rats in Exp. 3. Bars depict the number of 50-kHz calls emitted by the subject under study during test phases without acoustic presentation (nothing), presentation of noise (noise), artificial 50-kHz sine wave tones (50-kHz tones), and natural 50-kHz calls (50-kHz calls). Values reflect means±SEM per minute. Animals of all stimulus orders were collapsed, i.e. n = 36. Comparisons with $p<.05$ are marked with asterisks: *
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amplitude and frequency modulation are appetitive, since artificial 50-kHz sine wave tones also induced approach behavior. Despite the fact that natural 50-kHz calls tended to be more efficient in eliciting behavioral changes, amplitude and frequency modulation is apparently not a necessary prerequisite for the appetitive value of 50-kHz calls. Therefore, the present results are more in accordance with the assumption that a whole bundle of call features is responsible for the information, which is conveyed by such calls. We suggest, therefore, a compensatory model for 50-kHz calls, which states that the whole signal information is not lost when a specific call feature is missing, what would be predicted on the basis of the alternative conjunctive model.

Alternatively, one could assume that both, flat and frequency modulated calls, might be appetitive, but that the value of the latter is perhaps higher than that one of flat calls, a difference which is more likely to be detected in tests, like the one used by Burgdorf et al. [32], where the animal can actively chose between playback of different call varieties. Another explanation is that peak frequency rather than frequency-modulation is critical for the appetitive value of 50-kHz calls, since Burgdorf et al. [32] showed that frequency-modulated and flat calls also differ in their peak frequency. In the present study, only the amplitude and frequency modulation of calls was removed, but mean peak frequency remained unchanged, meaning that the 50-kHz sine wave tones used here had a peak frequency, which is typical for frequency-modulated calls. Actually, Brudzynksi [64] has suggested that, apart from call number, peak frequency is involved in coding the quantitative aspect of the sign function of 50-kHz calls, since peak frequency can be modulated by pharmacological agents, like glutamate [65].

Juvenile rats respond more strongly to 50-kHz calls than adult rats

Furthermore, we found that effects on overt behavior were more pronounced in juvenile rats than in adult rats. This age-related difference is even more impressive, when considering the relatively small number of young animals and the fact that the effect was evident irrespective of whether 22-kHz calls were presented in the same test or not. The difference in approach behavior between juvenile and adult rats is possibly reflecting a decrease in social interest in function of ageing. In fact, a reduced level of gregariousness among older individuals was consistently found in mammals. For instance, in a wide variety of primate species, aging leads to active withdrawal from social interactions and an increase in time spent alone [66–68]. Similar changes in function of age were also found in rats and mice. Thus, Salchner et al. [69] were able to show that aged rats spent considerably less time in active social interaction than young rats. Recently, Moles et al. [70] replicated this finding in mice. Interestingly, they did not only observe a decrease in the time spent investigating the partner, but also in the number of USV.

Furthermore, the stronger overt behavioral response in juvenile rats is in accordance with observations that 50-kHz calls occur predominantly in juvenile rats [37]. However, it remains unclear why young animals do not vocalize at all during playback of 50-kHz calls, whereas adult rats displayed ultrasonic calling in response to playback. One point, which might be of relevance in this context, is that the 50-kHz calls presented where emitted by adult rats, and it seems to be possible that call characteristics may convey information about age and status. Apart from these differences between juvenile and adult rats, it was observed that adult rats responded similarly to 50-kHz sine wave tones as to natural 50-kHz calls, whereas the response toward the artificial tones was not as strong as toward the natural calls in young animals. This difference might be due to a reduced acoustic sensitivity and plasticity in adult animals [71].

50-kHz ultrasonic calling and social approach

Rats are gregarious. For instance, two rats placed together in a large chamber spend substantially more time together than would be expected by chance, and are more attracted to other rats than to physical objects [72,73]. Obviously, social approach is crucial for establishing and maintaining relationships among individuals. The present findings indicate that the emission of 50-kHz calls is an important element in the evolvement of social relationships in rats. In fact, 50-kHz calls are typically emitted during social interactions, like reproductive behavior [21,23,25–28], juvenile play [19,20] and tickling [36–40]. That emission of 50-kHz calls is functional for these behaviors is indicated by studies showing that deafening or devocalizing rats can affect reproductive behavior [23,25,27,28,56,61] and reduces rough-and-tumble play [74]. Correspondingly, it was found that animals prefer to spend more time with other animals that vocalize a lot rather than with those that do not [75]. Furthermore, rats emit 50-kHz calls when entering areas where social contact has previously occurred [22,24,44,76,77]. Remarkably, the present findings nicely fit into earlier studies where it was shown that adult rats emit 50-kHz calls after separation from the cage mate, indicating that such calling serves to (re)establish or keep contact [43]. Similar conclusions can be drawn for mice, where USV was found during mating and social exploration [70,78–81]. Interestingly, Panksepp et al. [80] observed that high-frequency calling in mice is positively correlated with social investigation. Furthermore, Moles and ÒAmato [79] have shown that social investigation and the number of ultrasonic calls can be modulated by manipulating the attractiveness of the test partner. They have suggested, therefore, that ultrasonic calls facilitate proximity between animals, which helps to acquire relevant social information.

The study of social approach in laboratory animals can help to reveal biochemical, genetic and environmental factors underlying neuropsychiatric disorders such as depression, autism and Rett syndrome, since these are characterized, among others, by social deficits and loss of desire to engage in social interactions [82].

Bearing in mind the wealth of evidence implicating 50-kHz calls as a key element of social interactions in rats, it is noteworthy that the measurement of behavioral responses toward playback of 50-kHz calls provides a rather unique opportunity to study the determinants of social interest by using a standardized non-social test, i.e. without confounding effects of a partner. For instance, it is possible to model two core symptoms of the autistic syndrome, namely lack of social interest and communicative deficits [83,84].

Conclusion

The present findings clearly show that 50-kHz calls can induce approach behavior and ultrasonic calling in male rats. Thus, the hypothesis that such 50-kHz calls serve for communicative purposes, for example, to (re)establish or to keep contact with conspecifics, is supported.

SUPPORTING INFORMATION

Movie S1 Juvenile rat before and during playback of natural 50-kHz calls.

Found at: doi:10.1371/journal.pone.0001365.s001 (27.17 MB MPG)

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Author Contributions

Conceived and designed the experiments: MW RS. Performed the experiments: MW. Analyzed the data: MW. Contributed reagents/materials/analysis tools: RS. Wrote the paper: MW RS.

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Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain

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Abstract

Rodent ultrasonic vocalizations, which serve as sensitive measures in a number of relevant individual and social behaviours, have become increasingly interesting for biopsychological studies on emotion and motivation. Of these, high frequency (50-kHz) ultrasonic vocalizations can index a positive emotional state, and induce approach, whereas low frequency (22-kHz) ultrasonic vocalizations can induce avoidance and may index anxiety, since they are emitted during various unconditioned and conditioned aversive situations. While cholinergic and dopaminergic systems have been implicated, specific neural substrates that sub-serve these vocalization-dependent states remain to be elucidated. Using c-fos immunocytochemistry, we revealed neural activity in brain areas of naïve male Wistar rats in response to playback of 22-kHz and flat and frequency-modulated 50-kHz ultrasonic vocalizations. Presentation of background noise or no acoustic stimulus at all constituted the controls. Playback of 50-kHz ultrasonic vocalizations led to approach behaviour. Acoustically stimulated animals demonstrated differential activation in auditory areas, with a frequency-dependent activation in the auditory cortex. Specific forebrain, thalamic, hypothalamic and brainstem areas were also activated differentially. While 50-kHz playback induced sparse fos-like immunoreactivity in frontal association cortex, nucleus accumbens, thalamic parafascicular and paraventricular nuclei, 22-kHz playback elicited c-fos expression in the perirhinal cortex, amygdalar nuclei and the periaqueductal gray. This study unveils neural substrates that are activated during ultrasonic playback perception, which could sub-serve the affective states elicited by these vocalizations.

Keywords: Ultrasonic vocalization (USV); Playback; Aversive; Appetitive; Fos expression

The growing research interest in mammalian vocalization is concomitant with increasing interest in the underlying neural mechanisms, since vocalizations can index a great deal about brain, behaviour and the general state of the organism. In the rat, the impact that 22-kHz ultrasonic vocalizations (USVs) have on the receiver in terms of behaviour [27,5] and its neural substrates [1,2] have been studied. On the other hand, the effect of 50-kHz call presentation on behaviour has also been studied [9,33] and neural substrates have been suggested [17]. Playback of 22-kHz USVs can lead to avoidance or locomotor inhibition [5,10,33, but see 14,22], while 50-kHz calls can be appetitive [9], induced approach [33] and enhanced self-administration [9].

By far, as pharmacological studies have shown, it is the cholinergic [4] and dopaminergic [8] pathways that seem to affect overt behaviour and vocalization emission to a great extent, though other neurotransmitter systems also play a role [32]. While cholingeric pathways have been shown to underlie 22-kHz vocalization and the overt behaviour associated with a negative state, the dopaminergic system in the shell region of the nucleus accumbens is said to underlie 50-kHz calling and the positive state associated with it [8]. However, other studies have shown that the neural substrates involved in the initiation and production of these vocalizations are more complex [17].

Here, we use immediate early gene expression to screen for active brain regions in response to the playback of recorded ultrasonic calls. Immediate early genes are known to induce downstream cascades of gene-induction and represent cellular activity leading to protein synthesis. C-fos immunocytochemistry has served as a powerful tool for anatomical mapping of functional characteristics in complex systems such as the auditory brainstem pathways [15], in response to novel and familiar sounds [31], and in response to auditory stimuli that attain behavioural significance [28]. This would indicate that

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the response is not just reflecting auditory features of the stimulus, but also the salience of the stimulus, and this should be seen not only in auditory-relevant regions, but also regions associated with withdrawal and/or aversive behaviour, such as the periaqueductal gray and parts of the amygdala, and regions associated with positive affects, such as the ventral striatum.

Sixteen naïve male Wistar rats (HsdCpb:WU, Harlan-Winkelmann, Germany) weighing 100–124 g were procured, housed in groups of four in cages (Macrolon type IV) on Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland), and maintained in 12:12 h light/dark cycle (21–25 °C; 49–59% humidity). The animals were handled for 5 min on 3 consecutive days. On the 4th day, they were randomly assigned to four groups corresponding to the type of acoustic stimulus presented: no playback (arena-only); background noise, 22-kHz calls and 50-kHz calls. Then, they were removed from their home cages, isolated for 1 h, after which they were habituated to the test arena under red light (approx. 8 lux) for 1 h.

On the 5th day, animals were isolated for 1 h and then placed in the test arena, with playback of acoustic stimuli presented for 30 min. The testing arena (38 cm × 60 cm × 35 cm) consisted of two compartments (38 cm × 24 cm × 35 cm) joined by a central alley (38 cm × 12 cm × 35 cm). The two compartments had one side-wall replaced with a grid in front of which the loudspeaker was placed. The arena was wiped clean and the floor covered with fresh bedding each time. The recording room was devoid of any sound other than that from the recording equipment.

Acoustic stimuli, using hardware and frequency settings as described [33], were presented through an ultrasonic speaker (ScanSpeak, Avisoft Bioacoustics, Germany), placed 20 cm away from the test apparatus, with its position being changed from one compartment to the other for each animal. The calls presented had been recorded from a male Wistar rat while exploring a cage with scents from a cage mate (50-kHz), or from a rat that had received foot shocks (22-kHz). All stimuli [(a) 50-kHz of both, flat and frequency-modulated types [29], (b) long 22-kHz calls, and (c) background noise] were presented with a sampling rate of 192 kHz in 16-bit format, at ∼69 dB, with background noise presented at ∼50 dB, which corresponds to the background noise during playback of the other stimuli. Number of entries into the compartments and USVs emitted were recorded and analysed.

The animals remained in the testing arena for another 30 min, after which they were deeply anaesthetised and perfused transcardially with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, post-fixed and cryo-protected. Coronal sections of 30 μm were cut on a cryostat and subsequently processed for immunocytochemistry.

Briefly, sections were washed in 0.01 M phosphate buffered saline (PBS), rinsed in 0.2% Triton (PBS-T) detergent, endogenous peroxidase activity blocked with 0.3% hydrogen peroxide (H_2O_2), incubated in 5% normal goat serum (NGS-Vector S-1000), then transferred to c-fos antiserum (sc-52; Santacruz Biotech., 1:1000; 1% NGS) for 36–48 h. Sections were subsequently incubated in biotinylated goat anti-rabbit antiserum (1:100) followed by avidin–biotin–horseradish-peroxidase complex (Vector Elite PK-6101), and bound peroxidase visualised with 0.025% diaminobenzidine tetrahydrochloride (Sigma) and 0.06% H_2O_2.

Fos expression was screened qualitatively on a BX 61 Olympus microscope. Fos-positive cells were then quantified using Stereoinvestigator® (6.00-MicroBrightField Inc.) according to histologically defined criteria of the rat atlas [25]. Counting was done in a stipulated 0.25 mm × 0.25 mm square area on randomly selected sections from each brain. Photomicrographs were made using an Optronics digital camera MicroFire™ and worked on using Corel Draw (Corel Corp., 2000).

Experiments were carried out in accordance with the European Communities Council Directives, and permitted by the local animal ethics committee.

Behavioural results show that the 50-kHz group demonstrated significantly more locomotor activity during playback (total entries—arena-only: 53.25 ± 14.77; background: 56.25 ± 8.07; 22-kHz: 58.25 ± 11.18; 50-kHz: 98.75 ± 7.98; group means ± S.E.M.; $p = 0.049$; Kruskal–Wallis H-test), which was mainly directed to the compartment with the loud speaker (number of entries—arena-only: 14.00 ± 4.02; background: 13.75 ± 2.02; 22-kHz: 14.00 ± 2.80; 50-kHz: 27.75 ± 2.87; $p = 0.032$). Entries into the compartment without the loud speaker did not differ significantly (arena-only: 12.75 ± 3.50; background: 14.50 ± 2.02; 22-kHz: 15.50 ± 2.90; 50-kHz: 20.75 ± 2.78; $p = 0.336$). While no 22-kHz calls were emitted by any of the groups, some 50-kHz calls were detected in all groups—arena-only: 0.41 ± 0.25; background: 0.075 ± 0.028; 22-kHz: 0.083 ± 0.052; 50-kHz: 0.21 ± 0.088 (means calls/min ± S.E.M.; $p = 0.210$).

Fos-like immunoreactivity was confined to the nuclei of activated cells, which could be easily distinguished from background (Fig. 1). Basal expression was observed in arena-only animals in the olfactory lobes, piriform cortex, dorsal thalamus, lateral habenular nuclei (Fig. 1D), septal areas and some hypothalamic nuclei. Since differential fos-like immunoreactivity was observed in the four groups, 35 regions of interest (Fig. 2) were selected to further quantify the differences (Table 1).

Compared to the arena-only condition, an upregulation of fos-like immunoreactivity was observed in the acoustically stimulated groups in various cortical areas, such as the auditory, motor, frontal association, temporal association and ectorhinal cortices. Activation was also detected in the nucleus accumbens shell region, in the lateral septum and in the dorso-medial periaqueductal gray. Significant differences between 22-kHz and 50-kHz groups were observed in the frontal and perirhinal cortices, basolateral and lateral amygdala, paraventricular thalamic nucleus and dorso-medial periaqueductal gray. While activation in the 22-kHz group was observed in the basolateral, lateral and medial parts of the amygdala and the perirhinal cortex, the 50-kHz group demonstrated some activation in the accumbens core and shell regions, the anterior cingulate and frontal association cortices.

Areas in the auditory pathway were labelled to varied extents, with sparse labelling in the inferior colliculus and moderate to dense labelling in the primary and secondary areas of the auditory cortex (AC). In the 22-kHz group, cells in the central nucleus of the inferior colliculus were

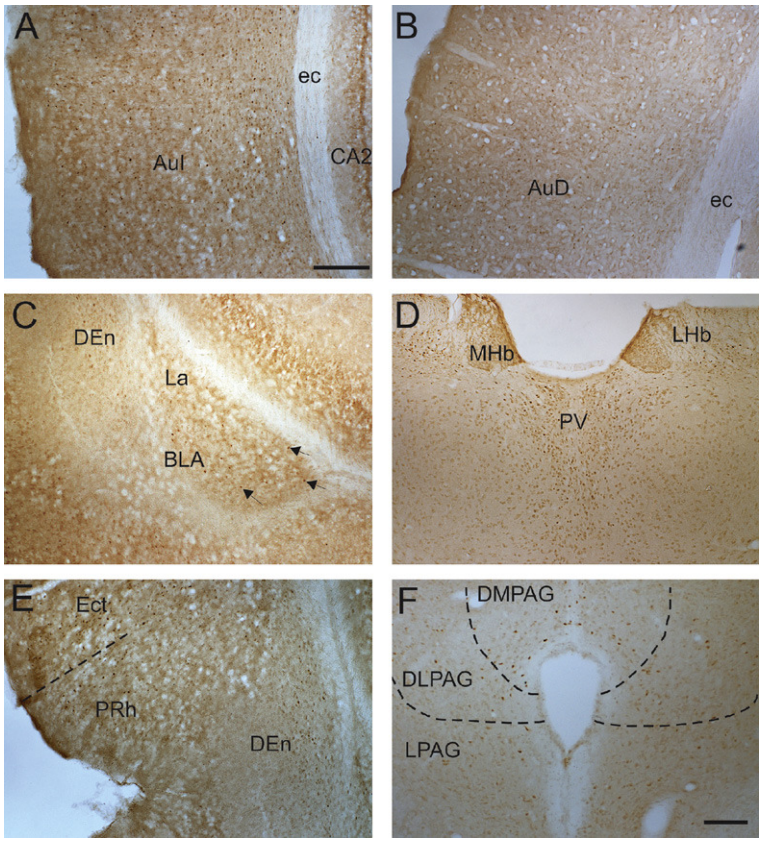

Fig. 1. Representative photomicrographs of fos-like immunoreactivity in response to playback of 22-kHz (left panel) and 50-kHz (right panel) ultrasonic vocalizations in male rats. (A) Fos expressing cells in the primary auditory area (AuI); (B) fos activation in the secondary auditory area (AuD); (C) few labelled cells in the lateral (La) and basolateral (BLA) amygdala, an expression not observed in the 50-kHz playback condition; (D) lateral habenular (LHb) and paraventricular (PV) nuclei; (E) fos labelling in the ectorhinal (Ect) and perirhinal (PRh) cortices; (F) activated cells in different sub-divisions of the periaqueductal gray (PAG; DM, dorso-medial; DL, dorso-lateral; L, lateral); fos expression seen in (D) and (F) was observed to varied extents in all four groups. Scale bar = 250 μm (*F*: 200 μm). Other abbreviations: CA2, field CA2 of hippocampus; ec, external capsule; DEn, endopiriform nucleus; MHb, medial habenular nucleus.

observed to be obliquely labelled across the nucleus, while there was very sparse fos-like immunoreactivity in the 50-kHz group.

Differential fos expression in AC was observed in response to playback of vocalizations of different frequencies. Labelling appeared either in discrete clusters in frontal AuD and AuV, or outspread through layers II–VI in AuI (Fig. 3). Hemispheric lateralization was also evident in the AC, with the left hemisphere showing higher activation. 22-kHz animals demonstrated dense fos expression in the primary auditory area (AuI, Fig. 1A) and in ventral (AuV) and dorsal (AuD) secondary auditory areas, while 50-kHz showed more c-fos activation in the frontal AuD (Fig. 1B) and AuV areas, and less in the AuI area. The temporal association cortex, ectorhinal cortex and to a certain extent the perirhinal cortex (Fig. 1E) were labelled in response to 22-kHz calls. The expression was lower in the 50-kHz group, except in the temporal association cortex, where it was on comparable levels.

In the amygdala, the basolateral and lateral nuclei contained a few scattered labelled cells in the 22-kHz group (Fig. 1C). In the 50-kHz group, sparse fos expression was observed in the central amygdala. Few fos expressing cells were observed in the medial shell region of the nucleus accumbens in the 50-kHz group, a pattern also observed in the arena-only group. In more caudal sections, few scattered nuclei were observed in the ventral core region.

The hypothalamus demonstrated differential fos expression in all groups. Parts of the pre-optic and lateral hypothalamus were labelled. In addition, the medial forebrain bundle, the ventral pallidum, and the parafascicular nuclei located just dorsal to the fornix in the thalamus demonstrated fos-positive cells in the 50-kHz group, but not in any other. In the rest of the brain stem, activation in sub-regions of the periaqueductal gray (Fig. 1F) was evident in all four groups to varied extents. While arena-only animals showed the least followed by 50-kHz animals, background and 22-kHz groups showed comparable expression. The pontine nuclei demonstrated comparable activation in response to 22- and 50-kHz playback.

Arena-only controls showed some fos expression. This activation is not due to novelty, as the animals had been habituated. It represents basal fos expression that exists in olfactory regions, visual cortex and a few other areas. All groups also demonstrated some thalamic, hypothalamic, and septal activation.

In the playback groups, cortical auditory regions were activated more in the left than the right hemisphere. This result is in line with previous evidence obtained in mice, where hemispheric lateralization in auditory cortex processing [13], and left hemisphere dominance in auditory perception and recognition

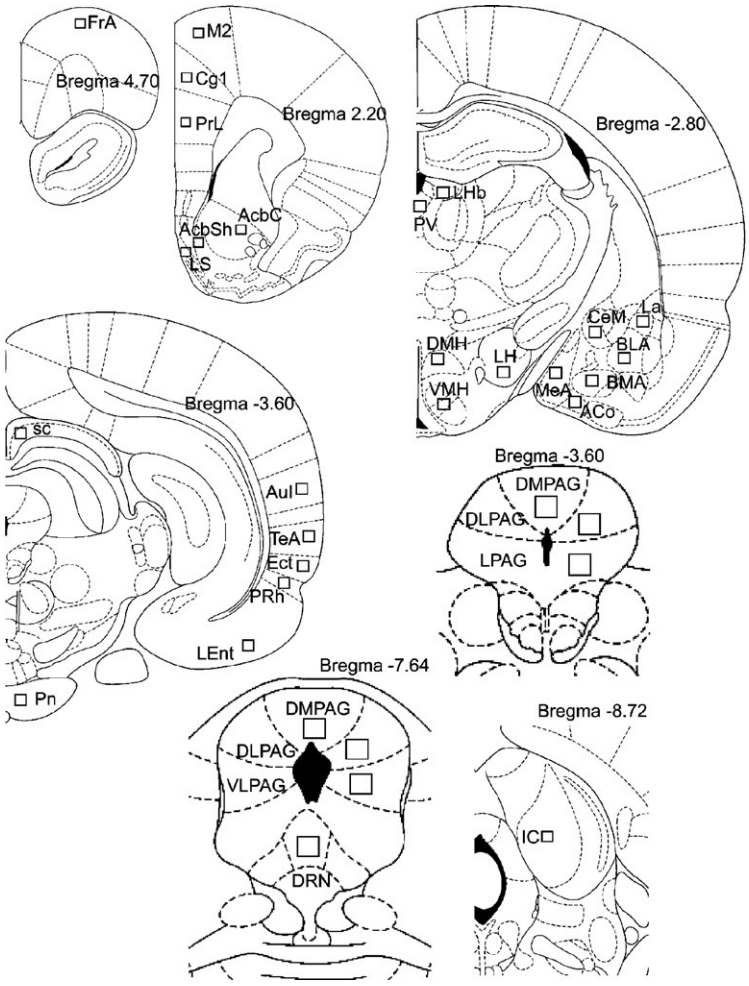

Fig. 2. Schematic diagrams of frontal sections of the rat brain from the Paxinos and Watson atlas, showing the 35 areas in which fos expression was quantified (Table 1). Open squares indicate the position of the 0.25 mm × 0.25 mm grid drawn to scale within which cell nuclei stained with fos were counted. For abbreviations, see Table 1.

was shown using c-fos mapping [16]. The fact that differential fos expression was observed here in the AuI, AuD and AuV areas could reflect a representation of the different frequencies perceived. While tonotopic fields AI and AAF with a high to low frequency gradient constitute the core [23,12], dorsally-, ventrally- and posteriorly located fields constitute the belt [23,26] of the auditory cortex. The more frontal fos expression observed in response to 50-kHz calls fits well with the topography of the high frequency area in AI and AAF [12,26], while fos expressing neurons found in clusters in the belt or secondary auditory areas could indicate processing at a higher level [18]. The increase in fos activation in the 22-kHz group could be due to the intensity, and the duration of the aversive acoustic stimulus, which can produce a spread of neuronal activation and an enlarging of tonotopic bands [28].

Functionally, 22-kHz calls are said to play an important role as alarm calls [3], and previous work has shown that such calls can lead to avoidance behaviour [5,10,33]. Such avoidance could not be detected here, which may be due to differences in the type of environment or behavioural measures. Nevertheless, presentation of 22-kHz calls led to neuronal activation in parts of the amygdala, albeit sparsely. While the lateral, basolateral and

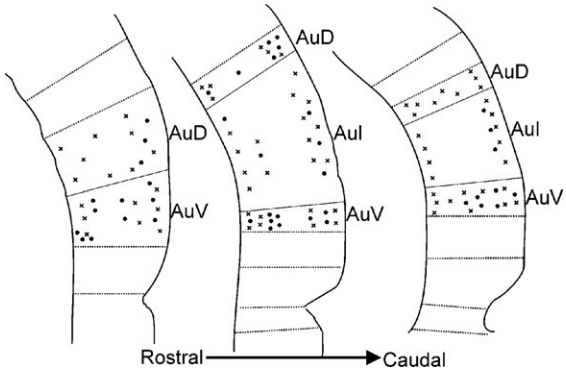

Fig. 3. Schematic representation of fos-like immunoreactivity in frontal sections of the auditory cortex. Crosses denote c-fos induction in response to playback of 22-kHz and circles to 50-kHz vocalizations. Shown is the activation in the left auditory cortex. The activation is attenuated in the right auditory cortex, cells being confined to the dorsal sub-division (AuD) in the 50-kHz group, while the activation is more spread out in the 22-kHz group. Rostral, Bregma −3.14 mm; caudal, Bregma −5.60 mm according to the Paxinos and Watson atlas.

Table 1
Number of fos-positive cells (mean ± S.E.M.) counted within a 0.25 mm × 0.25 mm square in 35 brain regions

Region	Bregma (mm)	Arena ($n=4$)	Background ($n=4$)	22-kHz ($n=4$)	50-kHz ($n=4$)	p values (H-test)
Auditory system						
Inferior colliculus (IC)	−7.80 to −8.80	8.05 ± 0.25	8.6 ± 1.5	4.3 ± 0.57*	4.5 ± 0.53*	0.005
Primary aud. cortex L (AuI)	−3.60 to −6.30	4.8 ± 1.12	7.35 ± 0.94	17.2 ± 0.96*,#	5.1 ± 0.66#	0.002
Primary aud. cortex R (AuI)	−3.60 to −6.30	5.15 ± 1.31	9.3 ± 0.97	14.3 ± 2.5	7.9 ± 2.78	0.108
Forebrain						
Frontal cortex (FrA)	3.70 to 1.70	0 ± 0	0.45 ± 0.33	0.35 ± 0.35#	2.3 ± 0.13*,#	0.003
Perirhinal cortex L (PRh)	−3.60 to −6.30	1.8 ± 1.2	0.95 ± 0.56	2.0 ± 0.54	0.85 ± 0.53	0.544
Perirhinal cortex R (PRh)	−3.60 to −6.30	0.30 ± 0.30	4.45 ± 0.68*	2.15 ± 0.33*,#	0.3 ± 0.24#	0.000
Motor cortex (M2)	3.70 to 1.70	0.35 ± 0.35	2.35 ± 0.31	1.1 ± 0.80	1.7 ± 0.69	0.105
Cingulate cortex (Cg1)	3.70 to 1.70	0.7 ± 0.41	1.0 ± 0.75	0.15 ± 0.15	1.85 ± 0.79	0.339
Temporal cortex (TeA)	−3.60 to −6.30	1.7 ± 0.98	5.95 ± 1.43	6.35 ± 0.59	6.45 ± 1.65	0.075
Ectorhinal cortex (Ect)	−3.60 to −6.30	1.25 ± 0.63	5.8 ± 2.15	4.5 ± 0.75*	2.15 ± 0.77	0.047
Prelimbic (PrL)	3.70 to 2.20	0.10 ± 0.10	0.90 ± 0.66	1.55 ± 0.94	2.1 ± 1.2	0.433
Entorhinal cortex (LEnt)	−5.20 to −6.30	1.2 ± 0.73	2.1 ± 2.1	0 ± 0	1.0 ± 0.87	0.107
Amygdala						
Ant. cortical amygdala (ACo)	−2.30 to −3.30	0.40 ± 0.28	1.8 ± 1.8	0 ± 0	0 ± 0	0.543
Medial amygdala (MeA)	−2.30 to −3.30	1.0 ± 0.51	4.0 ± 2.3	0.95 ± 0.95	0 ± 0	0.315
Basolateral amygdala (BLA)	−2.30 to −3.30	1.4 ± 0.36	0 ± 0*	3.9 ± 0.46*,#	0.9 ± 0.52#	0.000
Basomedial amygdala (BMA)	−2.30 to −3.30	0.7 ± 0.34	1.4 ± 1.4	0.25 ± 0.25	0 ± 0	0.235
Lateral amygdala (La)	−2.30 to −3.30	0.65 ± 0.65	0 ± 0	3.5 ± 0.75#	0.10 ± 0.10#	0.008
Central amygdala (CeM)	−2.30 to −3.30	2.9 ± 0.37	0 ± 0*	0.4 ± 0.4*	1.5 ± 0.29*	0.000
Basal ganglia						
Nuc. accumbens core (AcbC)	1.70 to 0.70	1.25 ± 0.36	0.45 ± 0.45	0.45 ± 0.45	2.55 ± 1.02	0.127
Nuc. accumbens shell (AcbSh)	1.70 to 0.70	0.8 ± 0.2	1.3 ± 0.60	1.4 ± 1.04	2.2 ± 1.2	0.783
Septum and hypothalamus						
Lateral septum (LS)	1.70 to 0.20	0.95 ± 0.95	4.2 ± 0.90	3.3 ± 3.3	1.5 ± 1.5	0.253
Lateral hypothalamus (LH)	−1.30 to 2.30	3.5 ± 1.57	7.0 ± 1.25	4.45 ± 1.13	2.45 ± 1.43	0.164
Ventromedial nucleus (VMH)	−2.80 to −3.30	6.75 ± 2.3	4.0 ± 2.4	1.8 ± 1.8	2.7 ± 1.6	0.317
Dorsomedial nucleus (DMH)	−2.80 to −3.30	5.35 ± 1.02	5.15 ± 0.94	6.4 ± 2.7	4.65 ± 1.73	0.986
Thalamus						
Paraventricular nucleus (PV)	−2.80 to −3.80	16.8 ± 2.64	11.95 ± 1.77	6.85 ± 0.45*,#	11.00 ± 1.75#	0.014
Lateral habenular nucleus (LHb)	−2.80 to −3.80	17.4 ± 3.49	10.8 ± 1.32	7.8 ± 1.81	7.4 ± 1.19*	0.023
Tectum						
Superior colliculus (SC)	−6.30 to −7.30	4.15 ± 1.35	6.15 ± 1.16	8.25 ± 0.66	4.10 ± 0.78	0.066
Periaqueductal gray						
Rostral dorso-medial (DMPAG)	−5.60 to −7.30	2.15 ± 0.26	3.9 ± 0.34*	4.6 ± 0.48*,#	2.5 ± 0.31#	0.001
Rostral dorso-lateral (DLPAG)	−5.60 to −7.30	2.7 ± 0.13	4.8 ± 0.74	4.0 ± 0.75	2.6 ± 0.53	0.096
Rostral lateral (LPAG)	−5.60 to −7.30	2.95 ± 0.78	1.85 ± 0.26	2.55 ± 0.58	1.95 ± 0.33	0.500
Caudal dorso-medial (DMPAG)	−7.64 to −8.72	2.35 ± 0.46	2.85 ± 0.92	3.25 ± 0.40	2.0 ± 0.49	0.506
Caudal dorso-lateral (DLPAG)	−7.64 to −8.72	2.2 ± 0.55	3.05 ± 0.41	4.1 ± 0.77	2.55 ± 0.3	0.206
Caudal ventro-lateral (VLPAG)	−7.64 to −8.72	1.95 ± 0.40	1.5 ± 0.13	2.05 ± 0.56	2.5 ± 0.17	0.281
Tegmentum						
Dorsal raphe nuclei (DRN)	−7.64 to −8.72	1.45 ± 0.26	3.75 ± 0.54*	0.20 ± 0.20*	0.4 ± 0.16*	0.000
Pontine nuclei (Pn)	−6.72 to −7.30	5.8 ± 0.50	3.75 ± 0.84	6.2 ± 2.19	6.55 ± 0.17	0.104

Group means were tested using the non-parametric analysis of variance Kruskal–Wallis test (H-test). Individual group differences were further tested using the Mann–Whitney U-test. Abbreviations: L, left; R, right; aud, auditory; ant, anterior; Nuc, nucleus; all others are mentioned in brackets. For the exact location of each of the densitometric sites, see Fig. 2.

* $p<0.05$ relative to arena-only group.

# $p<0.05$ between 22-kHz and 50-kHz playback groups.

central amygdalar nuclei have been implicated in fear conditioning, central amygdalar lesions specifically appear to block production of 22-kHz USVs and freezing [11]. Another cortical structure implicated in 22-kHz perception [1,22] is the perirhinal cortex, which was also activated here. This multimodal cortex has reciprocal connections with the amygdala [22], and the fact that some neurons respond with a different firing pattern to USVs than to continuous control tones, indicates that neurons in the perirhinal cortex respond to complex 22-kHz USVs [1,22].

The periaqueductal gray, which was activated to varied extents in all four groups, seems to play a central role in coordination of different subsystems required to produce emotional vocalizations [21]. While the lateral sub-division is said to play

an important role in defensive responses and in the production of USVs, the ventro-lateral sub-division, which showed fos expression only in the 22-kHz group, is said to be important for submission, but has no known role in the emission of USVs. In previous work [2], c-fos expression was more pronounced than what is observed here, which could be due to differences in signal presentation, or the type of antibodies used there.

50-kHz USVs are elevated by food rewards, sexual behaviour, rough-and-tumble play, experimenter-induced "tickling", drugs of abuse, and anticipation of rewarding electrical brain stimulation [24,7,32,29]. This led to the hypothesis that 50-kHz calls index positive affective states associated with specific brain sites, including ventral striatum and pallidum [20,8]. Interestingly, the 50-kHz group was the only one which demonstrated sparse to moderate fos expression in the ventral striatum, ventral pallidum, medial forebrain bundle and in the parafascicular thalamic nucleus. While the latter has been specifically implicated in juvenile play in rats [30], other brain areas, such as the inferior colliculus, dorsal periaqueductal gray, ventromedial hypothalamus, ventral striatum activated here also demonstrate enhanced c-fos mRNA during play behaviour in juvenile rats [17], a situation during which the rate of 50-kHz calls is increased [19,6].

Taken together, this study demonstrates differential early gene expression in diverse brain areas in response to playback of 22- and 50-kHz vocalizations. Some of these activations may index negative and positive affective states elicited by these different vocalizations, while others may indicate stimulus-specific processing, though it is clear that more studies are required to completely unravel the brain circuitries that underlie responses to conspecific calling in rodents.

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Studie VI

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Ultrasonic calling during fear conditioning in the rat: No evidence for an audience effect

Running headline: Audience effect on rat ultrasonic calling? – Wöhr/Schwarting

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Abstract

Rats emit 22-kHz ultrasonic vocalizations in aversive situations such as during cat exposure or fear conditioning. These calls are considered to be part of the animal's defensive repertoire and might serve as alarm signals for conspecifics. The aim of the present study was to test whether the emission of 22-kHz calls is affected by the social context during fear conditioning. Animals were tested in one of three experimental conditions, i.e. either alone, with an anesthetized conspecific, or with an active conspecific. In line with the hypothesized alarming function of 22-kHz calls, it was expected that the presence of a conspecific should potentiate the production of 22-kHz calls. The present results, however, show that the emission of 22-kHz calls during fear conditioning is clearly not potentiated by the presence of a conspecific; if at all, its presence had a mild attenuative effect on call rate. Also, call characteristics were similar in all three experimental conditions. Therefore, the present findings do not support the hypothesis that the sender is actively producing 22-kHz calls to warn conspecifics about danger.

Key words: 22-kHz call, alarm call, communication, fear conditioning, freezing, rat (rattus norvegicus), social behaviour, social buffering, ultrasonic vocalization (USV).

Introduction

Rats emit distinct types of ultrasonic vocalizations, which differ depending on animal age, the subject's current state, and environmental factors (for reviews see: Constantini & D'Amato, 2006; Knutson et al., 2002; Portfors, 2007). 22-kHz calls are emitted when rats are exposed to aversive situations, like intermale aggression (Kaltwasser, 1990a; Kroes et al. et al., 2007; Lore et al, 1976; Lehman & Adams, 1977; Sales 1972; Thomas et al., 1983), electric shocks (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005; Wöhr & Schwarting, in press), acoustic startle (Kaltwasser, 1990b; Kaltwasser, 1991), predators (Blanchard et al., 1990; Blanchard et al., 1991; Blanchard et al., 1992; Shepherd et al., 1992; for review see: Litvin et al., 2007), handling (Brudzynski & Ociepa, 1992), social isolation (Francis, 1977), and drug withdrawal when prompted by mild startling stimuli (Barros & Miczek, 1996). Also, these calls can be observed as conditioned responses (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005; Wöhr & Schwarting, in press). Interestingly, anxiolytic drugs can reduce such vocalizations (Jelen et al., 2003; for review see: Sanchez, 2003). Accordingly, it was assumed that 22-kHz calls reflect a negative affective state akin anxiety (Jelen et al., 2003).

Furthermore, 22-kHz calls are considered to be part of the animal's defensive repertoire (Brudzynski, 2001), since they are closely associated with the freezing response to actual or potential threat (Choi & Brown, 2003; Wöhr et al., 2005; Wöhr & Schwarting, in press), and might serve as alarm calls for conspecifics (Blanchard et al., 1990; for review see: Litivin et al., 2007). In fact, it was shown that presentation of natural 22-kHz calls or 20-kHz sine wave tones can affect behaviour of the receiver. Dependent on the strain of the receiver, animals showed a reduction in locomotor activity (Brudzynski & Chiu, 1995; Burman et al., 2007; Commissaris et al., 2000; Neophytou et al., 2000; Sales, 1991; Wöhr & Schwarting, 2007), or bursts of running and jumping, which is characteristic of defensive behaviour (Beckett et al., 1996; Beckett et al., 1997; Commissaris et al., 1998; Commissaris et al., 2000; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Voits et al., 1999). These behavioural changes are accompanied by increased expression of c-fos, a marker for neural activity, in the periaqueductal grey and the amygdala (Beckett et al., 1997; Neophytou et al., 2000; Sadananda et al., 2008). Interestingly, ultrasound induced defensive behaviour can also efficiently be attenuated by anxiolytics (Beckett et al., 1996; Nicolas et al., 2007). Other studies, however, obtained no behavioural effect when presenting 22-kHz calls (Endres et al., 2007; Lindquist et al., 2004; Tankhiwale et al., 2007; Sadananda et al, 2008).

The hypothesis that 22-kHz calls serve as alarm calls to warn conspecifics is born out

a study of Blanchard et al. (1991), who showed that the production of 22-kHz calls in response to an predator is dependent on the presence of conspecifics, i.e. on the presence of an audience. Thus, the term "audience effect" was coined, which states that the production of vocal signals is not only sensitive to eliciting stimuli, but also to the caller's social context, i.e. the presence and identity of a listener. Such an audience effect was also demonstrated in birds (Evans & Marler, 1991; Evans & Marler, 1992; Gyger et al., 1986; Karakashian et al., 1988; Ridley et al., 2007; Sullivan, 1985) and other mammals, like primates (Cheney & Seyfarth, 1985; Wich & Sterck, 2003), prairie dogs (Hoogland, 1983; Hoogland, 1996) marmots (Blumstein et al., 1997), and mongoose (Roux et al., 2008). However, it has to be noted that the observed audience effect in rats might have been based on other factors than the absence or presence of an audience, since the animals tested differed not only therein, but also with regard to the test apparatus, test duration, and housing conditions (Blanchard et al., 1991). It is reasonable that these covarying factors could have affected calling behaviour. Thus, it was already shown that housing conditions can affect 22-kHz call emission (Inagaki, et al., 2004; Nunes Mamede Rosa et al., 2005; Tomazini et al., 2006).

The aim of the present study was to test whether the emission of 22-kHz calls is affected by the absence or presence of a conspecific. Here, a conventional fear conditioning paradigm was used, which was shown to induce 22-kHz calling before (Borta et al., 2006; Wöhr et al., 2005; Wöhr & Schwarting, in press). As usual, foot shocks were used as aversive stimuli, since their features, like intensity and duration, can precisely be determined. The emission of 22-kHz calls was compared in a highly standardized way between the following three different conditions: fear conditioning (1) without a conspecific, (2) with an anesthetized conspecific, and (3) with an active conspecific.

Materials and methods

Animals and housing

For this study, sixty naïve male Wistar rats (HsdCpb:WU, Harlan-Winkelmann, Borchen, Germany), weighing 200 – 224 g on delivery, were procured. They were housed in groups of 5 in Macrolon type IV cages (size: 380 x 200 x 590 mm, plus high stainless steel covers) bedded with Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland), and maintained in an animal room with a 12:12h light/dark cycle (lights on 07:00 – 19:00 h) at 21 – 25 °C (humidity: 45 – 70 %). Lab chow (Altromin, Lage, Germany) and water (0.0004 % HCl-solution) were available ad libitum. All animals were allowed to adjust to the housing conditions for about 2 weeks. Prior to testing, all animals were handled for 3 days in a

standardized way (5 min each day), and randomly divided into experimental rats, which underwent fear conditioning, and companion rats, which were present during fear conditioning but not conditioned themselves. In each cage, 3 rats were assigned to the test group and 2 rats to the companion group.

All experimental procedures were performed according to legal requirements of Germany and approved by the ethical committee of the local government (Regierungspräsidium Giessen, Germany). After this experiment, rats were used for further behavioural experiments outside the scope of this study.

Fear conditioning

Experimental setting

Fear conditioning was performed in a shock chamber (size: 335 x 350 x 380 mm) made of gray and transparent plastic walls. In one side wall, a grid (size: 265 x 155 mm; grids had a diameter of 5 mm and were spaced 5 mm apart) was assembled (Fig. 1). The roof and one other wall were made of transparent plastic to allow video observation. The shock chamber was equipped with a loudspeaker (diameter: 75 mm; Conrad Electronic, Hirschau, Germany), which was mounted in one wall 30 cm above the floor. The floor of the shock chamber was made of stainless steel rods (diameter: 5 mm) spaced 10 mm apart.

A second shock chamber, which was constructed in a mirror-inverted, but otherwise identical way, was placed nearby, so that the side walls with the grid were spaced 10 cm apart. Both shock chambers were illuminated with bright white light (about 200 lux) provided by two LED spots each (MR16 with 18 LEDs, diameter: 5 mm; Conrad Electronic), which were placed 170 mm above the shock chambers. A similar experimental setting was used before to demonstrate an audience effect on alarm calling in chickens (Evans & Marler, 1991; Evans & Marler, 1992).

Experimental procedure

A 3-day procedure was used. On the first day (termed habituation), each experimental rat was placed in the shock chamber for 11 min to measure baseline behaviour and possible vocalizations. On the second day (termed conditioning), it was placed again into the shock chamber for 11 min. After an initial phase of 3 min, where no tone or shock was given, the rat was exposed to six tone/shock pairings, each followed by an ISI of 60 s. As the conditioned stimulus (CS), a 3-kHz sine wave tone (generated with: SASLab Pro, version 4.2, Avisoft Bioacoustics, Berlin, Germany) was presented for 20 s. As the unconditioned stimulus (UCS)

a 0.5 mA scrambled shock (52 Hz, peak-to-peak amplitude 120 V) was used, which was administered by a stand-alone shocker (Med Associates, St. Albans, USA) during the last 500 ms of the tone. This shock intensity was selected based on an earlier study, where 0.5 mA turned out as the lowest shock intensity, which can induce unconditioned and conditioned ultrasonic calling in a reliable manner (Wöhr et al., 2005). Subjectively, the sensation evoked by this shock intensity in humans is comparable to a tingling. On the third day (termed testing), the rat was again placed into the shock chamber for 11 min. After an initial phase of 3 min, the tone, but no shock, was presented six times for 20 s each.

All behavioural tests were conducted between 09:00 – 17:00 h. Prior to each test, behavioural equipment was cleaned using a 0.1 % acetic acid solution followed by drying.

Independent variable

Experimental rats underwent fear conditioning either without a cage mate (NC, n = 12), with an anesthetized cage mate (XC, n = 12), or with an active cage mate (AC, n = 12) present in the adjacent shock chamber. These conditions were maintained throughout all three experimental days, i.e. habituation, conditioning, and testing. During conditioning, shocks were delivered only to the experimental rat, i.e. companion rats did not receive any shocks. Throughout the experiment, animals were kept together in Macrolon type IV cages, each cage containing five subjects, that is, one per condition, i.e. 1 AC experimental rat, 1 XC experimental rat, 1 NC experimental rat, and 2 companion rats, one, which was active during testing, and one, which was anesthetized during testing. Anaesthesia was performed by using ketaminhydrochloride (1.0 ml/kg; Ketavet, Pharmacia, Erlangen, Germany) and xylazin (0.5 ml/kg; Rompun, Bayer Health Care, Monheim, Germany). Anaesthesia lasted about 30 min and did not cause any signs of distress. Animals were allowed to recover in a Macrolon type III cage (size: 265 x 180 x 420 mm) with no other rat present. Thus, anesthetized animals were never in direct tactile contact with active animals, meaning that the active animals were not able to cause any harm to them.

Dependent variables

Recording and analysis of overt behaviour

Overt behaviour was monitored by two video cameras (high resolution colour camera with infrared, Conrad Electronic), which were placed about 420 mm away from the shock chambers, and fed into a DVD recorder (DVR-3100 S, Pioneer, Willich, Germany), or a video recorder (CVC SQPB, Panasonic, Hamburg, Germany), respectively. Immobility (duration of

phases without somatic mobility except respiratory activity), rearing (number of times an animal reared on its hind legs), and grooming (duration of face, body, and genital grooming movements) were scored from DVD or video by experienced observers. Interrater reliability of these observers was found to be high for all three measures (immobility: rho = 0.960, $p < 0.001$; rearing: rho = 0.943, $p < 0.001$; grooming: rho = 0.839, $p < 0.001$).

Recording and analysis of ultrasonic vocalization

Ultrasonic vocalizations were monitored by two UltraSoundGate Condenser Microphones (CM 16; Avisoft Bioacoustics) placed in the roof of the shock chambers 30 cm above the floor. Two microphones, one in each chamber, were used in order to identify the sender of 22-kHz calls. These microphones were sensitive to frequencies of 15-180 kHz with a flat frequency response (± 6 dB) between 25-140 kHz. They were connected via an Avisoft UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics), and were recorded with a sampling rate of 250,000 Hz in16 bit format.

For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT length, 100 % frame, Hamming window and 75 % time window overlap). Correspondingly, the spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. Audible calls were counted by an experienced observer.

22-kHz call detection was provided by an automatic threshold-based algorithm (threshold: -40 dB) and a hold-time mechanism (hold time: 20 ms). A lower-cut-off-frequency of 18 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. An experienced user checked the accuracy of call detection and obtained a 100 % concordance between automatic and behavioural detection. Then, various parameters, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element, were determined automatically. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. Temporal parameters determined included latency to call, call duration, total calling time, and the duration of intervals between subsequent calls. Based on interval duration between two calls, calls were divided into those starting a bout versus those within a bout. A bout was defined as a call, or a number of calls, which was separated from other calls by intervals longer than 320 ms, according to Van der Poel (1991; for details see: Wöhr et al., 2005). To describe the temporal patterning of call production, the

numbers of bouts and bout length, i.e. the number of calls within a bout, were examined. Additionally, calls were divided into calls emitted during tone presentations or calls emitted during ISIs. Finally, the total number of 22-kHz calls emitted was measured.

Statistical analysis

To determine call duration, bout length, peak frequency, and peak amplitude, the mean of each call parameter served as statistical unit for each subject. The numbers of animals used per group, were chosen to ensure sufficient statistical power for inferential statistics (power analysis: Cohen's f = 0.50; $\alpha = 0.10$, $\beta = 0.80$). Since several data sets were not normally distributed as indicated by Shapiro-Wilk-tests, non-parametric statistics were used. Wilcoxon-tests were used to test whether animals showed differences in their overt and calling behaviour dependent on test phases. For between-group comparisons, Kruskal-Wallis-tests were used, followed by Mann-Whitney-U-tests when appropriate. To test whether the number of vocalizing animals differs between treatments, chi²-tests were calculated. Finally, Spearman correlation coefficients were calculated to describe the relationship between immobility and calling. One experimental rat was excluded from analysis, since the anesthetized cage mate woke up during conditioning.

Results

Habituation

Overt behaviour

Descriptively, animals tested with an active cage mate tended to show more behavioural activity, but the statistical analysis did not yield evidence for a substantial difference, neither during min 1 – 3 of exposition to the novel environment (immobility: chi² = 2.257, p = 0.324; rearing: chi² = 1.033, p = 0.596; grooming: chi² = 3.386, p = 0.184; Fig. 2, left), nor during the following min 4 – 11 (immobility: chi² = 0.088, p = 0.957; rearing: chi² = 4.751, p = 0.093; grooming: chi² = 3.566, p = 0.168; see Fig. 2, right).

Ultrasonic vocalization

Mere exposure to the novel environment did not induce 22-kHz calling, neither during min 1 – 3 (Fig. 2, left), nor during min 4 – 11 (Fig. 2, right).

Conditioning

Overt behaviour

Min 1 – 3 (Fig. 2, left): Immobility was low and did not differ between the habituation day and the conditioning day in all three groups (all p-values > 0.100). However, in each group rearing activity decreased from the habituation day to the conditioning day (all p-values < 0.05), whereas grooming increased (all p-values < 0.05).

When comparing behaviour between groups during the initial min 1 – 3, no differences in immobility ($chi^2 = 0.888$, $p = 0.642$) and rearing ($chi^2 = 0.131$, $p = 0.936$) were obtained, but rats differed in grooming activity ($chi^2 = 6.488$, $p = 0.039$), since subjects, which were tested in the presence of an active cage mate, spent more time grooming than those tested in the presence of an anesthetized one ($Z = -2.339$, $p = 0.019$; all other p-values > 0.100).

Min 4 – 11 (Fig. 2, right): During shock delivery, rats displayed short bursts of activation, with startle movements, flinches and running, paralleled by some few audible calls (range: 0.38 – 2.00 per min; not shown in detail). With repeated shock delivery, the predominant response was an increase in immobility. Thus, rats of all three groups now showed more immobility than during corresponding periods of the habituation day (all p-values < 0.05). The increase in immobility was paralleled by decreases in rearing and grooming (all p-values < 0.05).

When comparing the three groups during shock delivery, no substantial differences were obtained (immobility: $chi^2 = 1.362$, $p = 0.506$; rearing: $chi^2 = 5.608$, $p = 0.061$; grooming: $chi^2 = 3.182$, $p = 0.204$).

Ultrasonic vocalization

Min 1 – 3 (Fig. 2, left): As on the habituation day, mere exposure to the shock chamber did not induce 22-kHz calling.

Min 4 – 11 (Fig. 2, right; Table 1): During shock delivery, however, 22-kHz vocalizations were detected in all three groups, namely in 10 out of 12 rats, which were tested in the absence of a cage mate, in all 11 rats, which were tested in the presence of an anesthetized cage mate, and in 7 out of 12 rats, which were tested in the presence of an active cage mate ($chi^2 = 1.271$, $p = 0.559$). Remarkably, in vocalizing animals, the latency to start calling was dependent on this context ($chi^2 = 6.543$, $p = 0.038$). Rats, which were tested in the absence of a cage mate, started earlier to emit 22-kHz calls than rats, which were tested in the presence of an anesthetized cage mate ($Z = -2.535$, $p = 0.010$; all other p-values > 0.100; when all animals are included in the analysis: NC: 418.53±37.18 s; XC: 459.45±27.29 s; AC: 517.53±39.30 s; $chi^2 = 5.301$, $p = 0.071$). However, call number and total calling time did not

differ between groups (chi^2 = 1.967, p = 0.374 and chi^2 = 1.619, p = 0.445, respectively). Furthermore, call characteristics (call duration: chi^2 = 1.736, p = 0.420; peak frequency: chi^2 = 0.059, p = 0.971; peak amplitude: chi^2 = 0.945, p = 0.623) and temporal patterning (bout number: chi^2 = 0.022, p = 0.989; bout length: chi^2 = 1.732, p = 0.421) were similar in all three conditions. A similar picture was obtained when calls were divided into those, which were emitted during tone presentation or during ISIs (not shown in detail).

When analyzing behaviour of the active companion group, which attended the shock-exposed rats, it was found that the companions showed a concomitant increase in immobility, but did not emit 22-kHz calls (not shown in detail). Remarkably, the number of 22-kHz calls emitted by the experimental rats was positively correlated with the time companion rats spent immobile (rho = 0.660, p = 0.020), whereas the time, which the experimental rats spent immobile, was not correlated with that of the companion rats (rho = 0.462, p = 0.121; see Fig. 3, left). Unlike 22-kHz calls, the number of audible calls emitted by experimental rats in response to shock delivery was not related to immobility of the companion rats (rho = -0.114, p = 0.723).

Correlation between immobility and 22-kHz calling

During shock delivery, immobility and 22-kHz calling of the experimental rats were positively correlated (rho = 0.384, p = 0.023), and correlation coefficients were similar in all three groups (rho = 0.413 – 0.518; see Fig. 4, left).

Testing

Overt behaviour

Min 1 – 3 (Fig. 2, left): When re-exposed to the context on the subsequent testing day, the level of immobility was higher in all three groups than during the initial 3 min periods of the habituation day and the conditioning day (all p-values < 0.05), indicating conditioned fear evoked by the context. Conversely, the number of rearings and the time spent grooming were lower in all three groups than during corresponding periods of the prior days (all p-values < 0.05).

When comparing overt behaviour between the three groups, no substantial differences were obtained (immobility: chi^2 = 5.294, p = 0.072; rearing: chi^2 = 1.294, p = 0.524; grooming: chi^2 = 0.601, p = 0.741).

Min 4 – 11 (Fig. 2, right): During tone presentation, but irrespective of social context, the rats spent more time immobile than during the corresponding phases of the habituation

day (all p-values < 0.05), and even more than during the corresponding period of the conditioning day (XC: Z = -1.956, p = 0.054; all other p-values < 0.05), indicating conditioned fear evoked by context and CS. Rearing behaviour was lower in all three groups during tone presentation on the testing day than during that of the habituation day (all p-values < 0.05). However, a further reduction on the testing day in comparison to the corresponding period of the conditioning day was only evident in rats, which were tested in the absence of a cage mate (Z = -2.484, p = 0.012), but not in rats, which were tested in the presence of an anesthetized cage mate (Z = -0.352, p = 0.781), or an active cage mate (Z = -1.070, p = 0.308). Grooming was also lower in all three groups during tone presentation on the testing day than during the corresponding period of the habituation day (all p-values < 0.05), but not that of the conditioning day in all three groups (all p-values > 0.100).

When comparing overt behaviour between the three experimental groups, no differences were obtained (immobility: chi² = 0.915, p = 0.633; rearing: chi² = 0.571, p = 0.752; grooming: chi² = 0.392, p = 0.822).

Ultrasonic vocalization

Min 1 – 3 (Fig. 2, left): Mere exposure to the context where fear conditioning had taken place on the preceding day did not induce 22-kHz calling.

Min 4 – 11 (Fig. 2, right; Table 1): During tone presentation, however, 22-kHz calls were detected in all three groups, namely in 5 out of 12 rats, which were tested in the absence of a cage mate, in 3 of 11 rats, which were tested in the presence of an anesthetized cage mate, and 5 out of 12 rats, which were tested in the presence of an active cage mate (chi² = 0.421, p = 0.832). In line with the lower number of vocalizing animals, it was found that the number of calls emitted on the testing day was lower than that of the conditioning day in animals tested in the absence of a cage mate (Z = -2.701, p = 0.004) and in the presence of an anesthetized cage mate (Z = -2.535, p = 0.008), whereas there was no difference in animals tested with an active cage mate present (Z = -1.682, p = 0.102).

When comparing calling behaviour between groups, it was found that the latency to start calling (chi² = 2.488, p = 0.288), call number (chi² = 0.980, p = 0.613), and total calling time (chi² = 0.993, p = 0.609) were similar. Moreover, call characteristics were also similar in all three conditions (call duration: chi² = 0.826, p = 0.662; peak frequency: chi² = 4.941, p = 0.085; peak amplitude: chi² = 2.066, p = 0.356). Finally, no evidence for a different temporal patterning between groups was obtained (bout number: chi² = 2.198, p = 0.333; bout length: chi² = 1.465, p = 0.481). A similar picture was obtained when calls were divided into those

emitted during tone presentation or during ISIs (not shown in detail).

Companion rats showed an increase in immobility, but they did not emit 22-kHz calls (not shown in detail). In contrast to the conditioning day, the number of 22-kHz calls emitted by the experimental rats was not correlated with the time of immobility in the companion rats (rho = 0.359, p = 0.252), whereas now immobility of the experimental rats was positively correlated with that of the companion rats (rho = 0.799, p = 0.002; see Fig. 3, right).

Correlation between immobility and 22-kHz calling

During shock delivery, immobility and 22-kHz calling of the experimental rats were again positively correlated (rho = 0.390, p = 0.021), and correlation coefficients were low in XC and AC rats (rho = 0.237 and rho = 0.281, respectively), but relatively high in AC rats (rho = 0.616; see Fig. 4, right).

Discussion

The present fear conditioning paradigm was efficient to induce conditioned responses in overt behaviour and ultrasonic calling, which is in line with previous studies (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005; Wöhr & Schwarting, in press). Also in accordance with previous studies, a positive correlation between immobility and 22-kHz calling was observed in experimental rats (Choi & Brown, 2003; Wöhr et al., 2005; Wöhr & Schwarting, in press).

No evidence for an audience effect

Irrespective of treatment, all rats showed overt behavioural patterns, which are typical for the acquisition, expression, and extinction of conditioned fear. Furthermore, immobility, rearing, and grooming did not differ between the three experimental groups during conditioning and extinction, that is, the overt behavioural responses to the situation's aversivenes were apparently not affected by the presence of a conspecific in the adjacent cage. One reason could be that the experimental subjects were not perceiving the cage mate present in the adjacent chamber, but this argument can be ruled out, since behavioural activity before the first shock was delivered, namely rearing and grooming, tended to be higher in animals tested with an active cage mate. However, only the difference in grooming during the first three minutes on the conditioning day reached significance. In contrast, evidence for overt behavioural differences between experimental groups was no longer obtained after the first shock exposures. Interestingly, shock experience in experimental rats seemed to affect

behaviour of the active companion, since it also became immobile. This change is probably not due to a mere habituation effect to repeated chamber exposure, since its degree was correlated to the rate of 22-kHz calls emitted by the adjacent shocked rats. This secondary finding shows that these animals were able to perceive the presence of a conspecific and its behaviour during and after shock delivery.

In contrast to what was expected, the presence of an anesthetized or active cage mate in the adjacent chamber did not enhance ultrasonic calling in the experimental rats. Since a spectrographic analysis of vocalizations was used, these findings are based on both, quantitative and qualitative call features. This methodological detail should not be neglected, since it has been suggested that rats may signal information not only in terms of call rate, but specific features, such as call duration or peak amplitude (Brudzynksi, 2005; Wöhr et al., 2005). The hypothesis of an audience effect, which had motivated the present study, was originally based on the finding that rats emit 22-kHz calls ("alarm calls") in response to a predator predominantly when conspecifics are present (Blanchard et al., 1991). Such an audience effect on alarm calling was also reported in birds (Evans & Marler, 1991; Evans & Marler, 1992; Gyger et al., 1986; Karakashian et al., 1988; Ridley et al., 2007; Sullivan, 1985) and several other mammal species (Cheney & Seyfarth, 1986; Wich & Sterck, 2003, Hoogland, 1983; Hoogland, 1996, Blumstein et al., 1997, Roux et al., 2008). The present findings, however, clearly show that fear conditioned 22-kHz calling in rats is not potentiated by the presence of a cage mate. Rather, they indicate that the presence of a cage mate has a mild alleviative effect. Thus, the number of vocalizing animals during shock delivery was lowest for the group of rats, where an active cage mate was present in the adjacent shock chamber. Furthermore, not only call likelihood, but also the latency to call was affected by the presence of a cage mate, since rats started earlier to emit 22-kHz calls when tested alone.

Reduced calling behaviour in the presence of a cage mate is in line with other studies, which showed that the presence of another rat can be anxiolytic, an effect called social buffering. Davitz & Mason (1955) already showed that rats display less immobility during a retention test after fear conditioning when they were tested together with another rat than when tested alone. Similar results were obtained for the open field by Latané & Glass (1968). These findings were recently confirmed, and it was shown that the presence of another rat attenuated not only behavioural responses, but also stress induced hyperthermia and c-fos expression in brain areas implicated in anxiety regulation (Kiyokawa et al., 2004; Kiyokawa et al., 2007). Also, it is known that the emission of 22-kHz calls can be inhibited by anxiolytics, like benzodiazepines (Jelen et al., 2003; for review see: Sanchez, 2003). Thus, the

present call reduction could reflect an anxiolytic effect. This effect, however, was rather moderate, which might be explained by the fact that in contrast to other studies (Davitz & Mason, 1955; Kiyokawa et al., 2004; Kiyokawa et al., 2007; Latané & Glass, 1968), the rats used here were not tested in the same chamber, i.e. had no direct and tactile contact to each other. Finally, it is conspicuous that the rats tested in the presence of an anesthetized conspecific showed less ultrasonic calling than rats tested alone, but more than rats tested in the presence of an active conspecific. This indicates that not only the presence of an active conspecific may act in an anxiolytic way, but also the presence of a conspecific per se. However, it has to be mentioned that encountering an anesthetized animal is a somehow unnatural situation. Also, it cannot be ruled out that the anesthetized subject was not only behaviourally inactive, but that its smell differs from active conspecifics, meaning that anesthetized animals may not only differ by the absence of some features, like activity, but may have some features in addition, like a certain smell.

Possible reasons for the absence of an audience effect

The fact that ultrasonic calling was not potentiated by the presence of a cage mate is in contrast to the hypothesis that 22-kHz calls serve as alarm calls. The putative role of such vocalizations in conspecific communication had been indicated by the following two observations: (1) 22-kHz calls occur predominantly in social contexts and (2) 22-kHz calls can induce behavioural changes in the recipients. The present findings will be discussed in light of these observations as follows.

(**1) 22-kHz calls occur predominantly in social contexts:** Juvenile and adult rats emit 22-kHz calls in a variety of social situations, most prominently intermale aggression (Kaltwasser, 1990; Kroes et al. et al., 2007; Lore et al, 1976; Lehman & Adams, 1977; Sales 1972; Thomas et al., 1983) and mating, in particular during the postejaculatory period (Adler & Anisko, 1979; Barfield & Geyer, 1972; Barfield & Geyer, 1975; Choi & Brown, 2003). The importance of the social context was furthermore highlighted by a study of Blanchard et al. (1991), who found that rats emit 22-kHz calls in response to a predator predominantly when conspecifics are present.

The present results are clearly in contrast to these former findings. First of all, 22-kHz calls occurred in the absence of other rats (NC group). This observation is in line with several other studies, where presence of another rat was not a prerequisite for 22-kHz call production. Thus, individually tested rats can emit 22-kHz calls during fear conditioning (Borta et al., 2006; Choi & Brown, 2003; Jelen et al., 2003; Wöhr et al., 2005; Wöhr & Schwarting, in

press), when startled (Kaltwasser, 1990b; Kaltwasser, 1991), or when handled by the experimenter (Brudzynski & Ociepa, 1992). Most importantly, Francis (1977) has shown that social isolation can act as an acute inducer of 22-kHz calling.

Secondly, the present fact that 22-kHz calling was not potentiated by a social context also contrasts with the observations by Blanchard et al. (1991). These inconsistencies might be due to several reasons. One striking difference between the two studies is the aversive stimulus used. In the present work, foot shocks were administered, whereas Blanchard et al. (1991) used a cat. Furthermore, the experimental setting differed in multiple ways. Here, shocks were administered in classical shock chambers, whereas Blanchard et al. (1991) used an open field or a visible burrow system, which consists of an open area connected to a burrow system. Finally, only male rats where used in the present study, whereas Blanchard et al. (1991) used mixed-sex colonies. One could assume that each of these factors may be at least partly be responsible for the different findings. For instance, it was shown that adult male vervet monkeys emitted more alarm calls in the presence of a female than a male (Cheney & Seyfarth, 1985), referring to the crucial factor nepotism (Blumstein et al., 1997; Cheney & Syfarth, 1985; Hoogland, 1983; Hoogland, 1996; Sherman, 1977). Also, it has to be pointed out that the social and non-social test conditions of Blanchard et al. (1991) differed not only regarding the absence or presence of conspecifics, but also with respect to the test apparatus, test duration, and housing conditions. It is reasonable to assume that these covarying factors could have affected the behaviour. Thus, it was already shown that isolated housing can reduce 22-kHz call emission in aversive situations (Inagaki, et al., 2004; Nunes Mamede Rosa et al., 2005; Tomazini et al., 2006). Interestingly, the single housed animals in the study of Blanchard et al. (1991) did not vocalize when exposed to a cat, whereas the social housed, but individually tested animals in two subsequent studies vocalized when exposed to a cat (Blanchard et al., 1992; Shepherd et al., 1992). It seems possible, therefore, that the audience effect in the study by Blanchard et al. (1991) is actually based on several different factors, of which two important ones are the absence or presence of cage mates during testing and housing conditions before testing.

(2) 22-kHz calls can induce behavioural changes in the recipients: Blanchard et al. (1990) observed that the emission of 22-kHz calls in response to a cat can induce a profound and long-lasting set of defensive behaviours in conspecifics, which had not seen the cat themselves. Some of them even emitted ultrasonic calls, indicating that ultrasonic vocalizations are contagious. In the present study, the highly positive correlation between 22-kHz call emission of the experimental rat and the time, which the companion rats spent

immobile on the conditioning day, is in accordance with the observation that 22-kHz calls can induce behavioural changes in the recipients. However, evidence for the assumption that such calls are contagious was not provided, since no companion rat emitted 22-kHz calls. Possibly, the conspecifics in the study of Blanchard et al. (1990) emitted 22-kHz calls because they were able to detect the presence of the predator via sound or smell themselves, and not because 22-kHz calls of other rats were contagious.

In principle, it is possible that other factors than the emission of 22-kHz calls caused immobility in the recipient, since companion rats could not only hear the shocked ones, but since they also saw and smelled them. Indeed, it is known, for instance, that pheromones, secreted in aversive situations, can induce immobility (Kiyokawa et al., 2006). In line with an unknown, but causal third factor, it was observed in the present study that recipient's immobility on the testing day was no longer correlated with the number of 22-kHz calls emitted by the experimental rats, but with their immobility, indicating the role of other and perhaps even more potent inducers of defensive behaviour than 22-kHz calls. To rule out such possibly confounding factors, playback studies are helpful, since they do not require the social presence of the sender.

Such playback studies support the hypothesis that 22-kHz calls serve as alarm calls, since it was shown that the presentation of natural 22-kHz calls or 20-kHz sine wave tones can affect the behaviour of the receiver. Dependent on receiver strain, animals showed reduced locomotor activity (Brudzynski & Chiu, 1995; Burman et al., 2007; Commissaris et al., 2000; Neophytou et al., 2000; Sales, 1991; Wöhr & Schwarting, 2007) or bursts of running and jumping, which is characteristic of defensive behaviour (Beckett et al., 1996; Beckett et al., 1997; Commissaris et al., 1998; Commissaris et al., 2000; Finn et al., 2004; Neophytou et al., 2000; Nicolas et al., 2007; Voits et al., 1999). However, when critically reviewing the literature, the evidence in favour of a communicative function of 22-kHz calls is weak. Thus, it has to be noted that effects were only clearly evident when rather loud and artificial continuous sine wave tones were used (Beckett et al., 1996; Beckett et al., 1997; Commissaris et al., 1998; Commissaris et al., 2000; Finn et al., 2004; Nicolas, et al., 2007; Neophytou et al., 2000; Voits et al., 1999), whereas natural stimuli led to only weak effects (Brudzynski & Chiu, 1995; Burman et al., 2007; Sales, 1991; Wöhr & Schwarting, 2007). Also, some studies did not detect a behavioural response to playback of natural 22-kHz calls at all (Endres et al., 2007; Lindquist et al., 2004; Tankhiwale et al., 2007; Sadananda et al, 2008). Moreover, all studies, where the response to natural 22-kHz calls was compared with responses to other ultrasonic stimuli, did not observe a differential response to 22-kHz calls as

an unconditioned stimulus (Endres et al., 2007; Sales, 1991; Tankhiwale et al., 2007).

Ultrasonic calls as signals

It is well established that ultrasonic vocalizations in rats can serve as important social signals. Thus, several studies have demonstrated that pup ultrasonic calling can induce maternal search and retrieval behaviour (Allin & Banks, 1972; Wöhr & Schwarting, in press). Furthermore, it was shown that 50-kHz calls, which are emitted in appetitive situations, like rough-and-tumble play (Brunelli et al., 2006; Knutson et al., 1998) or tickling (Panksepp & Burgdorf, 2000; Schwarting et al., 2007), can elicit social approach (Wöhr & Schwarting, 2007). Against the background of these studies, it seems to be unlikely that 22-kHz calls do not have any signal value.

First of all, it has to be highlighted that the present lack of an audience effect in ultrasonic calling does not rule out the possibility that such effects may occur in other contexts. Further studies should include important variables such as nepotism and housing conditions in their experimental design. Moreover, Endres et al. (2007) have shown that reactivity to 22-kHz calls depend on previous learning experience. Specifically, experimentally naïve rats did not show overt behavioural changes in response to 22-kHz calls, but they quickly learned to associate an aversive event with 22-kHz calls, and were more reluctant to extinguish this memory than in the case of other types of ultrasonic stimuli. It is reasonable to assume that social housing conditions are critical for this learning to occur, as indicated by social isolation studies (Inagaki, et al., 2004; Nunes Mamede Rosa et al., 2005; Tomazini et al., 2006).

Secondly, it has to be highlighted that, although no evidence for an audience effect on ultrasonic calling was provided here, this does not rule out the possibility of a signalling function of 22-kHz calls. In favour of such a function, a high positive correlation between 22-kHz calls emitted and immobility of the recipient was found here, indicating that 22-kHz calls can act as alarm signals. Importantly, such an alarming function does not require that the sender has active control over call production.

Finally, one has to bear in mind, that the signal value of 22-kHz calls may relate to several biological functions, such as an emotional one, namely to carry information about the emitter's emotional state, or an alarming function, namely to warn conspecifics about external danger or threatening predators to deter further pursuit (for review see: Brudzynksi, 2005; Litvin et al., 2007; Shelley & Blumstein, 2005). One would solely expect a potentiation of 22-kHz call production in the presence of conspecifics when such calls are actually directed to

conspecifics, but not when they are directed to predators or other threats.

Conclusion

The present experiment shows that the emission of 22-kHz calls in aversively motivated situations, here fear conditioning, is not necessarily potentiated by the presence of a cage mate. Therefore, it does not support the hypothesis that 22-kHz calls serve an active alarming function, i.e. that the sender is actively producing such calls to warn conspecifics about danger. From these results, however, one should not conclude that 22-kHz calls do not convey important information for the recipient, or that 22-kHz call production is independent from the social context. Rather one should assume that their emission and salience depend on several and additional features, like nepotism, housing conditions, and previous learning experience.

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Figure legends:

Fig. 1: The experimental setting used to test whether the emission of 22-kHz calls is affected by the presence of a conspecific.

Fig. 2: Bar graphs depicting the time spent immobile (s), the number of rearings, the time spent grooming (s), and the number of 22-kHz calls during min 1 – 3 (left) or min 4 – 11 (right) on the habituation day, conditioning day, and testing day. Bars (means ± SEM) reflect groups of animals, which were tested in the absence of a cage mate (black), in the presence of an anesthetized cage mate (grey), or in the presence of an active cage mate (white).

Fig. 3: Scatter plots in the upper row depict the individual relationship between ultrasonic vocalization (22-kHz calls; n) emitted by experimental rats and overt behavior (immobility; s) displayed by companion rats in the fear conditioning paradigm during min 4 – 11 on the conditioning day (left) and the testing day (right). Scatter plots in the lower row depict the individual relationship between overt behavior (immobility; s) displayed by experimental rats and overt behavior (immobility; s) displayed by companion rats in the fear conditioning paradigm during min 4 – 11 on the conditioning day (left) and the testing day (right).

Fig. 4: Scatter plots depicting the individual relationship between ultrasonic vocalization (22-kHz calls; n) and overt behavior (immobility; s) displayed in the fear conditioning paradigm during min 4 – 11 on the conditioning day (left) and the testing day (right). Symbols reflect animals, which were tested in absence of a cage mate (black circles), in the presence of an anesthetized cage mate (grey circles), or in the presence of an active cage mate (white circles). Regression lines: solid - animals, which were tested in absence of a cage mate, dashed – animals tested in the presence of an anesthetized cage mate, and dotted – animals tested in the presence of an active cage mate.

Figures:

Fig. 1:

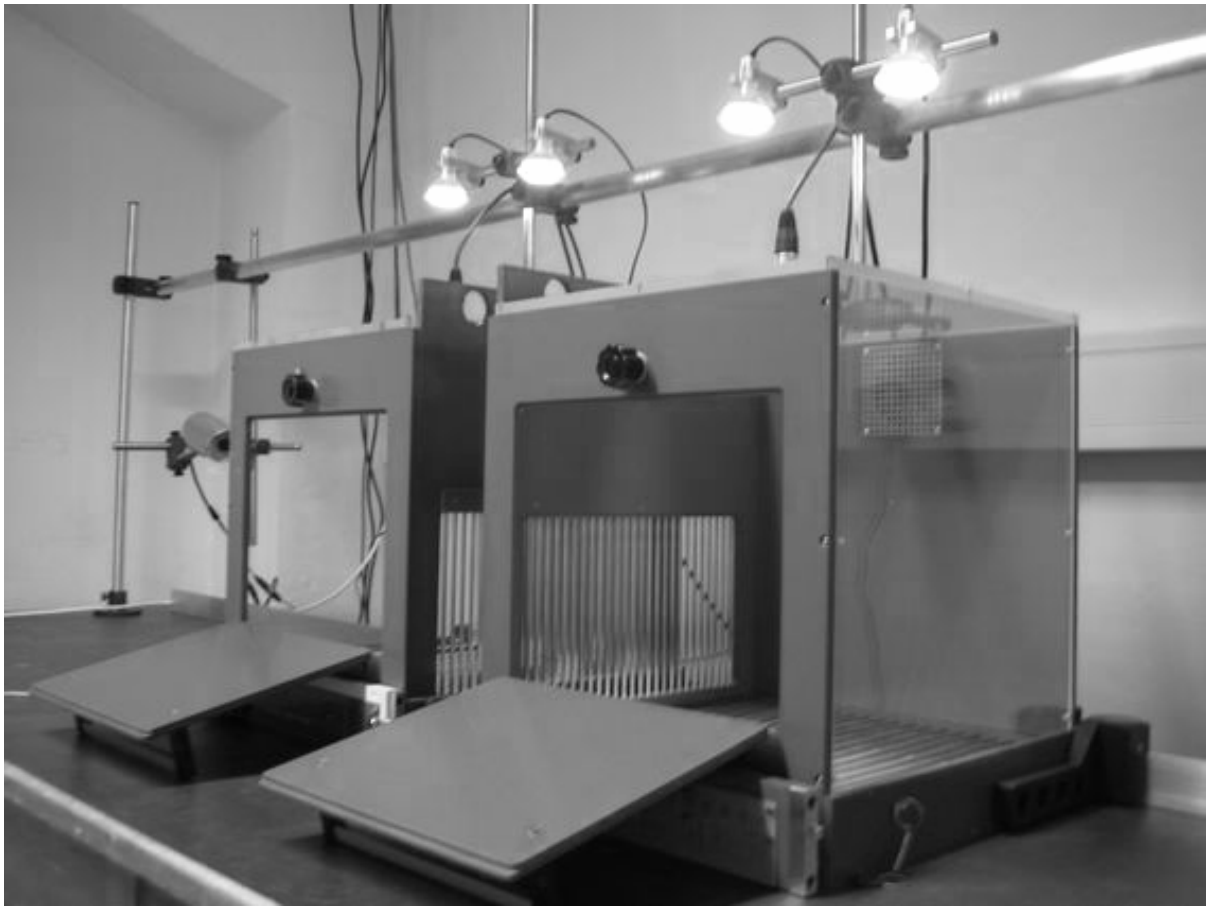


Fig. 2:

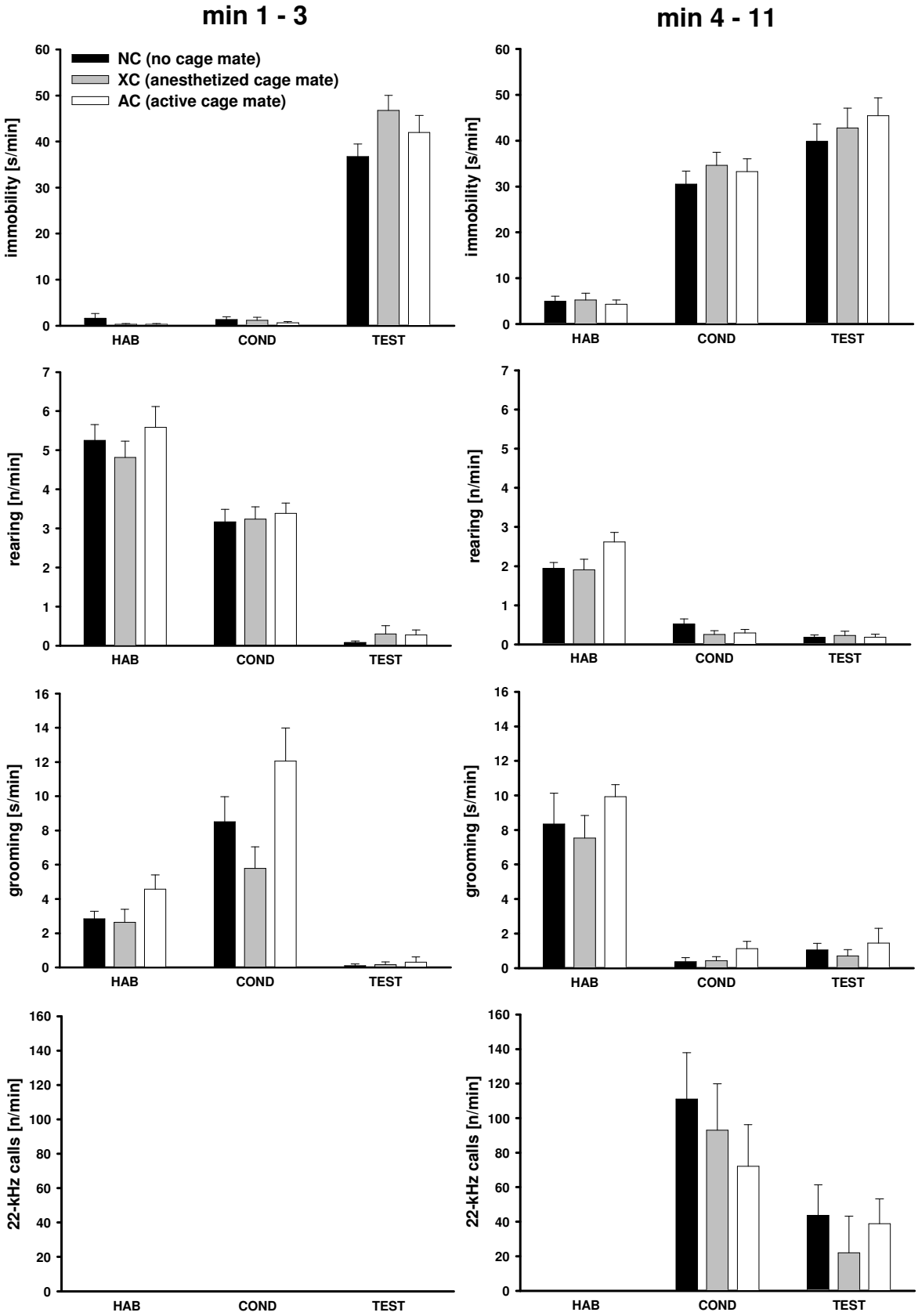

min 1 - 3
min 4 - 11
NC (no cage mate)
XC (anesthetized cage mate)
AC (active cage mate)
immobility [s/min]
rearing [n/min]
grooming [s/min]
22-kHz calls [n/min]
HAB
COND
TEST

Fig. 3:

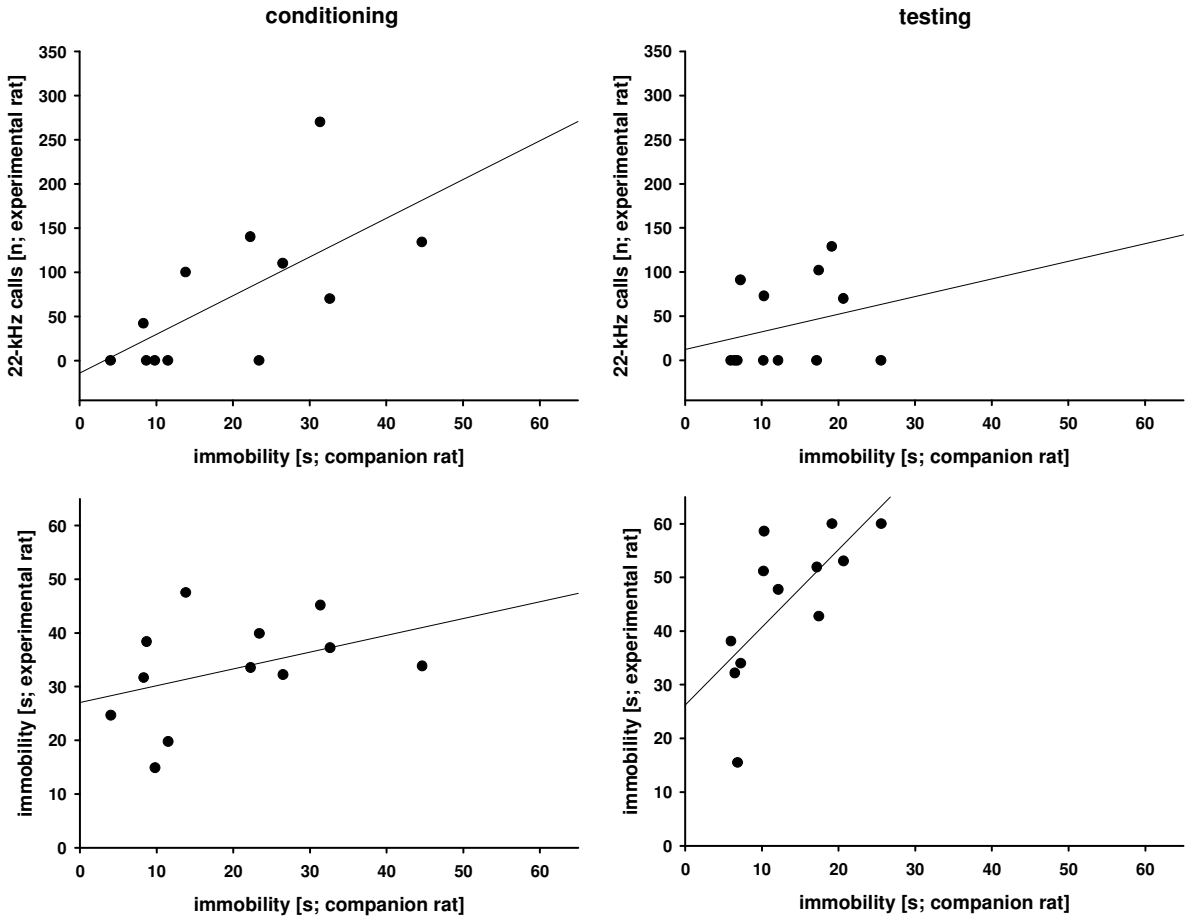
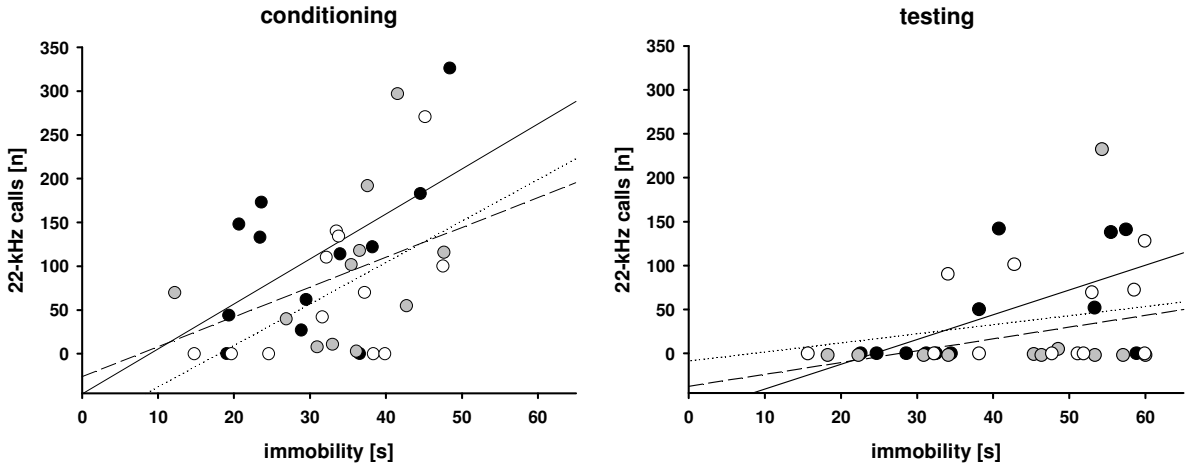

conditioning
testing
22-kHz calls [n; experimental rat]
immobility [s; companion rat]
immobility [s; experimental rat]
immobility [s; companion rat]

Fig. 4:


conditioning
testing
22-kHz calls [n]
immobility [s]
0
50
100
150
200
250
300
350
10
20
30
40
50
60

Tables:

Table 1:

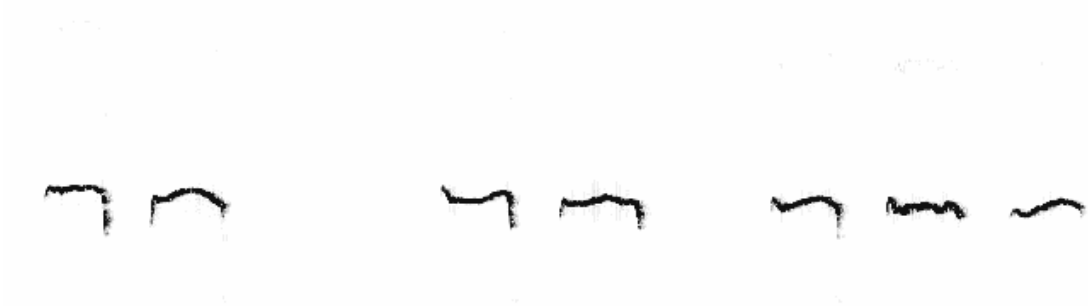
	conditioning					testing			
	NC	XC	AC			NC	XC	AC	
Vocalizing animals [n]	10/12	11/11	7/12	p = 0.559		5/12	3/11	5/12	p = 0.832
Latency to call [s]	370.24±21.73	459.45±27.29	415.76±26.67	p = 0.038		338.53±24.64	535.46±118.23	391.17±40.28	p = 0.288
Call number [n]	111.00±26.94	93.00±26.88	72.17±24.11	p = 0.374		43.58±17.73	22.00±21.21	38.75±14.44	p = 0.613
Total calling time [s]	106.36±22.91	83.80±21.89	71.04±21.63	p = 0.445		46.73±19.28	20.52±19.56	38.24±15.25	p = 0.609
Bout number [n]	34.58±7.24	22.36±5.11	23.50±6.97	p = 0.989		17.67±7.97	2.90±2.71	11.25±4.27	p = 0.333
Bout length [n]	3.29±0.42	4.34±0.97	3.32±0.60	p = 0.421		2.61±0.26	5.27±2.15	3.63±0.53	p = 0.481
Call duration [s]	1.09±0.12	0.94±0.05	1.06±0.07	p = 0.420		1.10±0.11	1.00±0.15	0.98±0.11	p = 0.662
Peak frequency [kHz]	23.87±0.38	23.78±0.28	23.76±0.41	p = 0.971		22.83±0.30	24.98±0.66	23.64±0.52	p = 0.085
Peak amplitude [dB]	68.43±1.63	70.35±1.61	70.69±1.51	p = 0.623		60.32±3.82	68.13±3.69	61.07±2.24	p = 0.356

Given are means± SEM. p-values reflect results of chi²-tests (vocalizing animals) or Kruskal-Wallis-tests (all other comparisons).

DISKUSSION

Die vorgelegten Arbeiten zeigen auf der einen Seite, dass soziale Faktoren, wie zum Beispiel die in den ersten Lebenstagen erfahrene maternale Zuwendung, einen erheblichen Einfluss auf die Produktion affektiver Ultraschallvokalisationen haben können. Auf der anderen Seite zeigen sie, dass Ultraschallvokalisationen ein potentes Mittel sind, um bestimmte Verhaltensweisen beim Empfänger, wie zum Beispiel soziale Annäherung, zu stimulieren.

In Übereinstimmung mit der Definition von Kommunikation (Bradbury & Vehrencamp, 1998), konnte gezeigt werden, dass alle drei Typen der Ultraschallvokalisation bei der Ratte Veränderungen im Empfänger auslösen, wobei entweder die Bereitstellung der Information dem Sender, wie im Falle der 40-kHz und der 50-kHz Rufe, oder der Zugang zur Information dem Empfänger, wie im Falle der 22-kHz Rufe, einen Nutzen bringt. Die Hypothese, die Ultraschallvokalisationen würden der innerartlichen Kommunikation dienen (Sales & Pye, 1974; Smith, 1979), konnte demnach bestätigt werden.



Bezüglich der kommunikativen Bedeutung von 40-kHz Vokalisationen konnte in den vorgelegten Studien gezeigt werden, dass diese bei der Mutter ein auf die Schallquelle hin ausgerichtetes Suchverhalten induzieren (Studie I). Die in Abwesenheit eines Jungtiers beobachteten starken Verhaltenseffekte belegen, dass der Geruch eines Jungtiers nicht, wie von Smotherman et al. (1974) vermutet, eine Voraussetzung dafür ist, dass Eintrageverhalten auftritt. Damit stehen die vorgelegten Beobachtungen in Einklang mit jenen von Allin und Banks (1972), die Suchverhalten auch ohne Präsenz von Geruch eines Jungtieres beobachten konnten. Auffällig ist jedoch die Tatsache, dass der in der vorgelegten Studie I beobachtete Effekt deutlich stärker ausgeprägt war als in der Studie von Allin und Banks (1972) - ein Umstand, der wahrscheinlich auf den zwischenzeitlich zu verzeichnenden technischen Fortschritt bei Systemen zur Präsentation von Ultraschallsignalen zurückzuführen sein dürfte. Gleichwohl schließen die vorgelegten Ergebnisse nicht aus, dass die zusätzliche Präsentation von Geruch eines Jungtiers zu einer Steigerung des Suchverhaltens führen könnte (Smotherman et al., 1974; 1978). Darüber hinaus stützt die Tatsache, dass die Mütter in der vorliegenden Arbeit nicht auf 40-kHz Sinustöne reagierten die von Brudzynski (1999) aufgestellte Hypothese, die Frequenzmodulation sei von zentraler Bedeutung für die

Lokalisation des Jungtiers, da Schall mit Frequenzveränderungen im Vergleich zu Schall mit gleich bleibender Frequenz leichter detektiert werden könne. Zusammenfassend zeigen die Ergebnisse also, dass 40-kHz Vokalisationen eine spezifische Signalfunktion besitzen. Das Aussenden von Ultraschallvokalisationen nutzt dem jungen Tier insofern, als dass die Mutter hierauf mit Such- und Eintrageverhalten reagiert, so dass das Jungtier nicht länger der unter Abwesenheit der Mutter drohenden Gefahren, wie Erfrieren, Verhungern oder von einem Räuber gefressen zu werden, ausgeliefert ist.

Für die Annahme, dass es sich hierbei um Signale des affektiven Zustands handelt, spricht die Beobachtung, dass die Auftretenshäufigkeit der 40-kHz Vokalisationen abhängig ist von der in den ersten Lebenstagen erfahrenen maternalen Pflege. Junge Ratten in Isolation emittieren in Abhängigkeit der erfahrenen maternalen Pflege unterschiedlich stark 40-kHz Vokalisationen (Studie I). Wie auch in einer Adoptionsstudie von Darnaudery et al. (2004) vokalisierten stark umsorgte Jungtiere seltener als wenig umsorgte. Diese Beobachtung steht in Einklang mit dem in vielen Studien nachgewiesenen anxiolytischen Effekt maternaler Pflege. Adulte Ratten, die von ihrer Mutter viel Zuwendung erfahren hatten, zeigen ein ausgeprägteres Explorationsverhalten und benötigen weniger Zeit, bis sie Futter in einer ihnen unbekannten Umgebung fressen als Tiere, die wenig Zuwendung erfahren hatten (Caldji et al., 1998; Francis et al. 1999). Umgekehrt zeigen die ersteren eine kürzer andauernde Verhaltensstarre nach Verabreichung eines elektrischen Schlags, eine geringer ausgeprägte Schreckreaktion nach Darbietung eines lauten Tons [*startle response*] und ein schwächer ausgeprägtes Bedecken aversiver Stimuli [*shock probe burying*] (Menard et al., 2004; Menard & Hakvoort, 2007; Zhang et al., 2005).

Kritisch muss allerdings angemerkt werden, dass anhand des korrelativen Ansatzes keine abschließenden Aussagen über Ursache und Wirkung getroffen werden können. Theoretisch erscheint es möglich, dass nicht die maternale Pflege ursächlich ist für das unterschiedlich stark ausgeprägte Vokalisationsverhalten, sondern dass Unterschiede zwischen den Jungtieren hinsichtlich des von ihnen gezeigten Vokalisationsverhaltens ursächlich sind für die unterschiedlich stark erfahrene maternale Pflege. Tatsächlich gibt es Untersuchungen, die zeigen konnten, dass 40-kHz Vokalisationen maternale Pflege auszulösen vermögen. Hier ist nicht nur das Eintrageverhalten (Studie I, Allin & Banks, 1972) zu nennen, sondern auch das maternale Lecken der Anogenitalregion der Jungtiere (Brouette-Lahlou et al., 1992) sowie Nestbauaktivität (Hashimoto et al., 2001; Noirot, 1972). Zwei Argumente sprechen jedoch gegen diese Annahme: Erstens würde man einen positiven Zusammenhang zwischen 40-kHz Vokalisationen und maternaler Pflege erwarten und nicht

den beobachteten negativen Zusammenhang. Zweitens belegt die an Mäusen vorgenommene Embryotransferstudie deutlich die Bedeutung von Umweltfaktoren für die Emission von isolations-induzierten Ultraschallvokalisationen (Studie II).

In Studie II konnte gezeigt werden, dass die Anzahl der emittierten Rufe stark von der Mutter beziehungsweise von der Interaktion zwischen Mutter und Jungtier abhängig ist. Dies steht in Einklang mit Befunden einer anderen Embryotransferstudie, wo die große Bedeutung epigenetischer Faktoren für das adulte angst-ähnliche Verhalten nachgewiesen werden konnte (Francis et al., 1999). In Adoptionsstudien (Priebe et al., 2005; Zaharia et al., 1996) und Kreuzungsstudien (Calatayud & Belzung, 2001; Calatayud et al., 2004) konnte gezeigt werden, dass von den zahlreichen epigenetischen Faktoren der maternalen Pflege eine Schlüsselstellung zukommt. Im Hinblick auf die isolations-induzierte Ultraschallvokalisation werden zukünftige Studien zu klären haben, welches Gewicht prä-, peri- und postnatale Faktoren jeweils unabhängig voneinander haben. Die beobachtete inverse Abhängigkeit von Ultraschallvokalisation und Eintrageverhalten weist jedoch auf eine große Bedeutung postnataler maternaler Faktoren hin. Eine von maternaler Pflege abhängige Beziehung zwischen isolations-induzierter Ultraschallvokalisation und Eintrageverhalten der Mutter konnte bereits D'Amato et al. (2005) in einer Adoptionsstudie aufzeigen. In Einklang mit der Vermutung, dass für die beobachteten epigenetischen Effekte Unterschiede in der maternalen Pflege ursächlich sind, ist die bemerkenswerte Übereinstimmung hinsichtlich der betroffenen Rufparameter bei Maus (Studie II) und Ratte (Studie I). Trotz unterschiedlicher experimenteller Vorgehensweisen erwiesen sich Rufanzahl und Lautstärke der Rufe in beiden Fällen als sensitiv für Veränderungen in der frühkindlichen Umwelt. Dies ist auch insofern interessant, als dass diese beiden Parameter bei adulten Tieren die Aversivität der Situation widerspiegeln (Wöhr et al., 2005). Eine Klärung der Frage, ob es sich bei den die Veränderungen im Vokalisationsverhalten bedingenden Umweltfaktoren wirklich um maternale Pflege handelt, könnte beispielsweise im Zuge von Kreuzungsstudien ermittelt werden. Hierbei müssten Tiere beider Mäusestämme miteinander verpaart werden, so dass Mischlinge erzielt würden, die dann jeweils von einer Mutter der beiden Mäusestämme aufgezogen werden (Calatayud & Belzung, 2001; Calatayud et al., 2004). Unter Verwendung von Kontrolltieren würde dies ferner helfen, den Erbgang der genetischen Komponenten aufzuklären.

Auf eine Bedeutung des Genotyps für die Anzahl der isolations-induzierten Ultraschallvokalisationen verweist in Übereinstimmung mit der Literatur (Hahn et al., 1987; 1997; 1998; Hahn & Schanz, 2002; Roubertoux et al., 1996; Thornton et al., 2005) die

Interaktion zwischen genetischen Hintergrund und Mutter. Rufparameter, wie Frequenz und Frequenzmodulation, waren sogar allein vom Genotyp abhängig. Ein bemerkenswerter Unterschied zwischen den beiden Mäusestämmen ist die Tatsache, dass B6JOla Mäuse eine Spontanmutation auf Chromosom 6 aufweisen, die zu einem Fehlen des Proteins alpha-Synuclein führte (Chen et al., 2002; Siegmund et al., 2005; Specht & Schoepfer, 2001; 2004). Er erscheint möglich, dass das Fehlen von alpha-Synuclein die Rufproduktion beeinflusst haben könnte. Bekanntermaßen wirkt sich alpha-Synuclein regulativ auf die dopaminerge Transmission aus (Abeliovich et al., 2000; Oksman et al., 2006), welche gemäß pharmakologischer Untersuchungen (Cuomo et al., 1987; Dastur et al., 1999; Kehoe & Boylan, 1992; Muller et al., 2005) und Zuchtstudien (Brunelli & Hofer, 2007) in der Produktion von isolations-induzierten Ultraschallvokalisationen involviert ist. Darüber hinaus konnten Muller et al. (2008) zeigen, dass Dopamin im Nucleus accumbens von besonderer Relevanz für die durch soziale Faktoren normalerweise induzierten Veränderungen der Emission von 40-kHz Rufe ist. So führt eine Verabreichung des D2/D3-Dopamin-Agonisten Quinpirol in den Nucleus accumbens zu einer Blockierung der normalerweise durch kurzen maternalen Kontakt induzierten Potentierung im Rufverhalten. Bemerkenswerterweise wurde kürzlich gezeigt, dass die Dopaminaktivität im Nucleus accumbens von hoher Bedeutung für die Entwicklung sozialer Bindungen ist (Aragona et al., 2003). Kritisch muss allerdings angemerkt werden, dass die vorgefundenen Unterschiede zwischen den Mäusestämmen nicht spezifisch auf das Fehlen des Proteins alpha-Synuclein zurückgeführt werden können, da die Spontanmutation zum Verlust mehrerer Gene führte (Specht & Schoepfer, 2004). Um die exakte Bedeutung von alpha-Synuclein für die Emission von isolations-induzierten Ultraschallvokalisationen aufzuklären, sollten alpha-Synuclein-Knock-Out Mäuse hinsichtlich ihres Rufverhaltens untersucht werden.

Zusammenfassend kann geurteilt werden, dass die isolations-induzierte Ultraschallvokalisationen bei Maus und Ratte potente Mittel sind, um maternale Pflege zu stimulieren, selbst aber trotz bedeutsamer genetischer Prädisposition zu erheblichen Teilen von dieser beeinflusst werden.

Bezüglich der kommunikativen Bedeutung von 22-kHz Vokalisationen konnte in den vorgelegten Studien gezeigt werden, dass diese beim Empfänger angst-ähnliches Verhalten auszulösen vermögen. Sowohl die künstliche Präsentation von 22-kHz Rufen (Studie IV) als auch 22-kHz Rufe, die von einem Tier selbst akut emittiert wurden (Studie VI), lösten lokomotorische Inhibition aus. Ferner konnte gezeigt werden, dass 22-kHz Rufe Hirnstrukturen aktivieren, wie Amygdala und zentrales Höhlengrau (Studie V), die an der Regulation von Angst und Furcht beteiligt sind (LeDoux, 2000). Diese Befunde stimmen mit der Hypothese überein, nach welcher diese Rufe eine Alarmfunktion besitzen (Blanchard et al., 1991).

Tatsächlich konnte in mehreren Studien unter Verwendung natürlicher 22-kHz Vokalisationen eine lokomotorische Inhibition beobachtet werden (Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Sales, 1991). Wie in der vorgelegten Arbeit waren die Verhaltenseffekte jedoch schwach ausgeprägt. Eine mögliche Ursache hierfür könnte sein, dass in allen Untersuchungen eine Absenkung einer sowieso nicht allzu stark ausgeprägten Spontanlokomotion erfasst wurde. Es würde sich daher in künftigen Untersuchungen anbieten, zu prüfen, inwiefern ein stark ausgeprägtes aktives Verhalten durch die Präsentation

von 22-kHz Rufen gehemmt werden kann. Die bislang vorgefundene Schwäche der Verhaltensreaktion und die Tatsache, dass in anderen Untersuchungen überhaupt keine Änderungen des Verhaltens zu beobachten war (Bang et al., im Druck; Lindquist et al., 2004; Tankhiwale et al., 2007), legt aktuell zweierlei nahe:

Erstens kann vermutet werden, dass die unter Verwendung künstlicher 20-kHz Sinustöne zu beobachtenden starken Verhaltensänderungen (Beckett et al., 1996; 1997; Commissaris et al., 1998; 2000; Finn et al., 2004; Nicolas, et al., 2007; Neophytou et al., 2000; Voits et al., 1999) auf andere Merkmale als die Frequenz und der hierin potentiell kodierten Bedeutsamkeit zurückzuführen sind. Tatsächlich konnten Commissaris et al. (2000) zeigen, dass Sinustöne mit anderen Frequenzen, wie etwa 7 kHz oder 12 kHz, sogar effektiver sind. Es erscheint daher wahrscheinlich, dass die hohe Lautstärke der präsentierten Stimuli für deren stark aversiven Charakter verantwortlich ist. So wurden die 20-kHz Sinustöne teilweise mit über 100 dB präsentiert (Commissaris et al., 2000; Voits et al., 1999). Auch war die beobachtete Verhaltensreaktion positiv mit der Lautstärke der Stimuli assoziiert (Commissaris et al., 2000).

Zweitens kann vermutet werden, dass natürliche 22-kHz Vokalisationen nicht per se zu Flucht- und Vermeidensreaktionen führen, sondern beim Tier möglicherweise lediglich einen Zustand erhöhter Wachsamkeit induzieren. Dieser Zustand erhöhter Wachsamkeit könnte die Entdeckung eines Fressfeindes erleichtern oder auch die Bildung von Assoziation zwischen einem aversiven Ereignis und den damit einhergehenden situativen Merkmalen fördern. So konnten Endres et al. (2007) im Rahmen einer klassischen Furchtkonditionierung beispielsweise zeigen, dass Ratten unter Verwendung von 22-kHz Rufen als CS eine ausgesprochen langsame Furchtextinktion zeigen, obwohl diese zuvor nicht per se mehr Verhaltensstarre auslösten als verschiedene Kontrollsignale. Ratten zeigten in Reaktion auf 22-kHz Rufe, welche zuvor mit einem elektrischen Schlag gepaart dargeboten wurden, über mehrere Extinktionsphasen auch noch nach über einer Woche eine deutlicher ausgeprägte Verhaltensstarre als in Folge der Darbietung von ebenfalls zuvor mit einem elektrischen Schlag gepaarten Kontrollsignale. In Erweiterung dieser Befunde konnten Bang et al. (2008) mittels differentieller Furchtkonditionierung eine asymmetrische Stimulusgeneralisierung nachweisen. Wurden beispielsweise 22-kHz Rufe (CS^+), nicht aber 50-kHz Rufe (CS^-), mehrfach mit einem elektrischen Schlag gepaart dargeboten, so zeigen die Ratten eine konditionierte Furchtreaktion allein auf die 22-kHz Rufe. Wohingegen die Ratten auf 22-kHz und 50-kHz Rufe eine konditionierte Furchtreaktion zeigen, wenn 50-kHz Rufe (CS^+), nicht aber 22-kHz Rufe (CS^-), mit einem elektrischen Schlag gepaart dargeboten wurden. Die

Ratten wiesen also einen Diskriminationsbias auf, welcher als Resultat biologischer Prädisposition [*biological preparedness*] aufgefasst werden kann (Seligman, 1970, 1971).

Diese Prädisposition hinsichtlich der Effektivität des Erlernens von Furchtreaktionen weist auf die Bedeutung der Verwendung natürlicher Stimuli mit sozialer Signalfunktion hin. In zukünftigen Untersuchungen erscheint es daher vielversprechend zu sein, zu prüfen, inwiefern die 22-kHz Rufe an einem sozialen Erlernen von Furcht beteiligt sind. Dies gilt im Besonderen angesichts der positiven Korrelation zwischen 22-kHz Rufen, welche vom Experimentaltier während der Furchtkonditionierung emittiert wurden, und der im beobachtenden Tier aufgetretenen Verhaltensstarre (Studie VI). Nachgewiesenermaßen spielt nicht nur beim Menschen das Modelllernen eine bedeutende Rolle für den Erwerb von Furcht (Gerull & Rapee, 2002; Olsson & Phelps, 2004), sondern auch bei Affen (Cook & Mineka, 1989; Cook et al., 1985; Mineka et al., 1984) und Mäusen (Kavaliers et al., 2001; zur Übersicht siehe: Olsson & Phelps, 2007). Bezüglich der sozialen Transmission von Furcht bei der Ratte liegen jedoch widersprüchliche Ergebnisse vor. So konnten Lore et al. (1971) beobachten, dass Ratten die Flamme einer Kerze vermeiden, nachdem sie beobachtet hatten, wie sich ein Artgenosse verbrannte. Zu ähnlichen Ergebnissen gelangte del Russ (1975) unter Verwendung einer aktiven Vermeidungsaufgabe. White und Galef (1998) hingegen beobachteten keine ausgeprägtere Vermeidung eines elektrischen Kontakts bei Tieren, die einen Artgenossen beobachtet hatten, welcher einen elektrischen Schlag hierüber verabreicht bekam. Ein bemerkenswerter Unterschied zwischen den Studien ist, dass die Tiere in der Studie von Lore et al. (1971) und del Russo (1975) ihre Artgenossen hören konnten, wohingegen eine Plexiglaswand in der Studie von White und Galef (1998) lediglich Sichtkontakt gewährte. Möglicherweise sind 22-kHz Rufe für die soziale Transmission von Furcht bei der Ratte daher von entscheidender Bedeutung. Interessanterweise konnte beim Menschen während des Erlernens von Furcht anhand eines sozialen Modells in jenen Hirnregionen eine starke Aktivität beobachtet werden (Olsson et al., 2007), die auch durch die Präsentation von 22-kHz Rufen aktiviert werden (Studie V).

In Studie V konnte gezeigt werden, dass 22-kHz Rufe den perirhinalen Cortex, den ectorihnalen Cortex, die Amygdala und das zentrale Höhlengrau aktivieren. Eine erhöhte Aktivität in der Amygdala und dem zentralem Höhlengrau wurde auch in Folge der Präsentation von 20-kHz Sinustönen induziert (Beckett et al., 1997; Neophytou et al., 2000). Außerdem konnte man in Läsionsstudien zeigen, dass der perirhinale Cortex an der Verarbeitung von 22-kHz Rufen beteiligt ist. Nach Ausschalten dieser Struktur lernten Tiere nicht länger, diese Vokalisationen (CS) mit einem elektrischen Schlag (US) zu assoziieren

(Lindquist et al., 2004). Einzelzellableitungen in dieser Region bestätigten die Beteiligung des peririhnalen Cortex an der Verarbeitung von 22-kHz Rufen (Allen et al., 2007; Furtak et al., 2007). Bemerkenswerterweise handelt es sich bei den an der Perzeption von 22-kHz Rufen beteiligten Strukturen auch um jene, die an der Produktion beteiligt sind. So zeigten Depaulis et al. (1992), dass eine pharmakologische Erregung des zentralen Höhlengraus zur Emission von 22-kHz Rufen führt. Ergänzend hierzu konnte beobachtet werden, dass agonistische Auseinandersetzungen beim unterlegenen Tier neben der Produktion von 22-kHz Rufen zu einer Veränderung der Genexpression im zentralen Höhlengrau führen (Kroes et al., 2007). Bezüglich der Amygdala konnte im Rahmen einer Furchtkonditionierungsstudie gezeigt werden, dass eine Läsion der Amygdala vor Durchführung der Konditionierung zu einem Ausbleiben von 22-kHz Rufen in Reaktion auf einen CS führte. Da jedoch 22-kHz Rufe in Reaktion auf einen US weiterhin auftraten, muss gefolgert werden, dass eine intakte Amygdala zwar nicht notwendig ist für die Emission von 22-kHz Rufen, aber von entscheidender Bedeutung für die Bildung einer Assoziation zwischen US und CS (Choi & Brown, 2003). Das Vorliegen einer erhöhten Aktivität in der Amygdala in Folge der Präsentation von 22-kHz Rufen (Studie V) stützt daher die Annahme, dass 22-kHz Vokalisationen einen Zustand erhöhter Wachsamkeit induzieren, welcher durch eine Erleichterung der Bildung von Assoziationen zwischen einem aversiven Ereignis und den damit einhergehenden situativen Merkmalen charakterisiert ist. Tatsächlich war die beobachtete Aktivität im Bereich der basolateralen und lateralen Amygdala am stärksten ausgeprägt – diese Regionen stellen die Inputzone der Amygdala dar, in der Informationen aus verschiedenen Sinnessystemen eintreffen (Pitkänen et al., 1997). Die Bedeutung dieser Regionen liegt in der Vermittlung der emotionalen Bewertung von zunächst neutralen Signalen der Umwelt, indem diese durch Kopplung mit Gefahrenreizen eine furchtauslösende Wirkung erhalten (LeDoux, 2000). Eine Erhöhung der Aktivität konnte hingegen nicht im Bereich der zentralen Amygdala beobachtet werden – dieser Teil der Amygdala stellt die Outputzone dar, von welcher aus die unterschiedlichen Bestandteile der Furchtreaktion angesteuert und orchestriert werden (LeDoux, 2000). Das Ausbleiben einer erhöhten Aktivierung im Bereich der zentralen Amygdala steht also in Einklang mit der Schwäche der durch die Präsentation von 22-kHz Rufen ausgelösten Verhaltenseffekte (Studie IV; Bang et al., im Druck; Brudzynski & Chiu, 1995; Burman et al., 2007; Endres et al., 2007; Lindquist et al., 2004; Sales, 1991; Tankhiwale et al., 2007).

Gleichwohl scheint die Beobachtung, dass 22-kHz Vokalisationen nicht vermehrt bei Anwesenheit eines Artgenossen auftreten (Studie VI), die postulierte Alarmfunktion in Frage

zu stellen. Eine Vielzahl von Tierarten emittieren nämlich vor allem dann Alarmrufe, wenn es Artgenossen vor einer Gefahr zu warnen gilt (Blumstein et al., 1997; Cheney & Seyfarth, 1985; Evans & Marler, 1991; Gyger et al., 1986; Hoogland, 1983; 1996; Karakashian et al., 1988; Ridley et al., 2007; Roux et al., 2008; Sullivan, 1985; Wich & Sterck, 2003). Darüber hinaus konnte gezeigt werden, dass die Produktion von Alarmrufen nicht allein von der Anwesenheit von Artgenossen abhängig ist, sondern auch vom Verwandtschaftsgrad zwischen Sender und Empfänger. Nah verwandte Artgenossen werden eher gewarnt als fern verwandte (Blumstein et al., 1997; Cheney & Seyfarth, 1985; Hoogland, 1983; 1996; Sherman, 1977).

Bei der Ratte lag bislang nur eine Untersuchung zur Bedeutung der Anwesenheit von Artgenossen vor. Blanchard et al. (1991) beobachteten, dass Ratten bei Konfrontation mit einer Katze vor allem dann 22-kHz-Rufe aussenden wenn Artgenossen anwesend sind. Kritisch muss hinsichtlich dieser Studie jedoch vermerkt werden, dass mit der An- beziehungsweise Abwesenheit von Artgenossen weitere Faktoren verändert wurden, wie beispielsweise die Testumgebung und die Haltungsbedingungen der Tiere. Es erscheint daher möglich, dass die beobachteten Effekte nicht in der An- beziehungsweise Abwesenheit der Artgenossen begründet sind, sondern auf diese kovariierten Faktoren zurückgeführt werden müssen. Tatsächlich konnte gezeigt werden, dass Haltungsbedingungen einen starken Einfluss auf die Emission von 22-kHz Rufen haben. So zeigten Inagaki et al. (2004), dass Ratten, welche nach der Trennung von der Mutter über sechs Monate hinweg allein gehalten wurden, in aversiven Situationen kaum 22-kHz-Rufe ausstießen, wohingegen in Paaren aufgewachsene Ratten in der gleichen Situation häufig vokalisierten. Die Beobachtung, dass isoliertes Aufwachsen die Emission von 22-kHz Rufen reduziert, wurde seither mehrfach bestätigt (Nunes Mamede Rosa et al., 2005; Tomazini et al., 2006).

Dennoch könnte die Tatsache, dass in der vorliegenden Arbeit im Gegensatz zu der Studie von Blanchard et al. (1991) keine Steigerung der 22-kHz Rufe in Anwesenheit eines Artgenossen auftrat, auch auf andere Faktoren zurückzuführen sein. Blanchard et al. (1991) testeten die Tiere in einer seminatürlichen Umgebung unter Anwesenheit von Tieren des anderen Geschlechts. Es erscheint durchaus möglich, dass die Anwesenheit von Tieren des anderen Geschlechts einen Einfluss auf die Rufproduktion in aversiven Situationen hat. So konnte beispielsweise bei der grünen Meerkatze beobachtet werden, dass Männchen eher Alarmrufe aussenden, wenn ein Weibchen in Gefahr ist als wenn ein Männchen in Gefahr ist (Cheney & Syfarth, 1985). In diesem Zusammenhang ist ferner zu bemerken, dass sich teilweise kürzlich geworfene Jungtiere in den von Blanchard et al. (1991) untersuchten

Sozialverbänden befanden. Gemäß der Annahme, dass die Anwesenheit von nah verwandten Artgenossen die Produktion von Alarmrufen begünstigt (Blumstein et al., 1997; Cheney & Seyfarth, 1985; Hoogland, 1983; 1996; Sherman, 1977), sollte dies hier in besonderem Maße der Fall sein. In zukünftigen Untersuchungen sollten daher nah verwandte Tiere im hier genutzten Paradigma getestet werden. Auf Grundlage der vorhandenen Daten muss jedoch aktuell geurteilt werden, dass eine Steigerung der Alarmvokalisationen bei Anwesenheit von Artgenossen in der Ratte unter streng kontrollierten Bedingungen nicht bestätigt werden konnte. Dies widerspricht einer Alarmfunktion von 22-kHz Rufen nicht per se, sondern zeigt auf, dass die Produktion von Alarmrufen bei der Ratte wahrscheinlich nicht aktiv gesteuert wird.

Abschließend muss jedoch angemerkt werde, dass die vorgelegten Befunde auch mit der Hypothese in Einklang zu bringen sind, wonach die 22-kHz Vokalisationen dazu dienen im innerartlichen Kampf durch Signalisieren der Unterlegenheit das überlegene Tier von weiteren Angriffen abzuhalten (Lehman & Adams, 1977; Lore et al, 1976; Sales et al., 1974). Diese Hypothese basiert auf der Annahme, dass die durch 22-kHz Rufe induzierte Hemmung lokomotorischer Aktivität vom unterlegenen Tier dazu genutzt wird, um zu fliehen. Grundsätzlich sind also alle Studien, die eine durch 22-kHz Rufe verursachte lokomotorische Hemmung beobachten konnten, vereinbar mit dieser Annahme. Die vorgelegten Arbeiten können diese Hypothese nur insofern indirekt entkräften, als dass die beobachtete Aktivierung in Amygdala und zentralen Höhlengrau (Studie V) eher in Übereinstimmung mit einer Alarmfuktion zu bringen ist. Schlagenstes Argument gegen die Hypothese, dass diese Rufe aggressives Verhalten hemmen, ist jedoch die Tatsache, dass unterlege Tiere, die operativ devokalisiert wurden, nicht häufiger angegriffen werden als Tiere, die vokalisieren können (Thomas et al., 1983). Dass die Tiere überhaupt bei innerartlichen Kämpfen 22-kHz Rufe produzieren (Kaltwasser, 1990a; Kroes et al. et al., 2007; Lehman & Adams, 1977; Lore et al, 1976; Sales 1972a; Thomas et al., 1983), spricht wiederum gegen eine aktive Kontrolle des Vokalisationsverhaltens und verweist darauf, dass die Tiere in aversiven Situationen generell, das heißt unabhängig von der An- beziehungsweise Abwesenheit von Artgenossen, 22-kHz Rufe emittieren. Dies erklärt auch, warum beispielsweise langanhaltende soziale Isolation 22-kHz Vokalisationen auslöst (Francis, 1972) – eine Situation, die eben genau durch die Abwesenheit von Artgenossen charakterisiert ist.

Gegen die Annahme, dass 22-kHz Vokalisationen generell einen negativen affektiven Zustand reflektieren, scheint jedoch der Befund zu sprechen, dass maternale Fürsorge sich zwar reduzierend auf die Emission von 40-kHz Rufen auswirkt, aber positiv auf die Emission

von 22-kHz Rufen (Studie I). Bemerkenswerterweise ist jedoch die Emission von 40-kHz Rufen mit einer ausgeprägten lokomotorischen Aktivität assoziiert (Studie I; Hofer & Shair, 1978), wohingegen die Emission von 22-kHz Rufen stark mit der vom Tier in aversiven Situationen gezeigten Verhaltensstarre kovariiert (Studie I; Burgdorf et al., im Druck; Choi & Brown, 2003; Wöhr et al., 2005). Diese Beobachtungen legen nahe, dass maternale Fürsorge nicht die Auftretenswahrscheinlichkeit angst-ähnlichen Verhaltens per se verändert, sondern das Verhältnis zwischen aktiven und passiven Bewältigungsstrategien mit welchen das Tier auf aversive Situationen reagiert. Übereinstimmend hierzu konnte in einer weiteren Untersuchung zur Furchtkonditionierung nicht die erwartete negative Korrelation zwischen maternaler Pflege und Verhaltensstarre beobachtet werden (Bagot & Meaney, 2005).

Zusammenfassend kann daher die Hypothese formuliert werden, dass 22-kHz Vokalisationen in aversiven Situationen spontan, das heißt nicht aktiv gesteuert, als Teil eines passiven Stressbewältigungsstils auftreten, wobei sie dennoch eine Signalfunktion für potentielle Empfänger besitzen, bei welchem sie einen Zustand von Wachsamkeit induzieren.

Bezüglich der kommunikativen Bedeutung von 50-kHz Vokalisationen konnte in den vorgelegten Studien gezeigt werden, dass diese in Übereinstimmung mit der Literatur nicht allein in appetitiven Situationen auftreten, wie während des Spiels (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998), dessen experimenteller Nachahmung durch das Kitzeln (Burgdorf & Panksepp, 2001; Burgdorf et al., im Druck; Mällo et al., 2007; Panksepp & Burgdorf, 1999; 2000; 2003; Schwarting et al., 2007) oder der Paarung (Barfield et al., 1979; Bialy et al., 2000; Burgdorf et al., im Druck; Geyer & Barfield, 1978; McGinnis & Vakulenko, 2003; McIntosh et al., 1978; Sales, 1972b; White & Barfield, 1990; White et al., 1990), sondern auch in neutralen oder gar aversiven Situationen (Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Knutson et al., 1999; Schwarting et al., 2007; Thompson et al., 2006; Wintink & Brudzynski, 2001).

Die Beobachtung, dass Tiere auch nach Trennung von einem Artgenossen vokalisieren (Studie III) kann nur schwerlich mit der affektiven Hypothese, nach welcher diese Rufe allein einen positiven affektiven Zustand reflektieren, erklärt werden. Die Tatsache, dass die Tiere nach Trennung voneinander rufen, wie auch die Beobachtung, dass die Rufe vor allem kurz nach der Trennung und danach immer seltener auftreten (Studie III) steht jedoch im Einklang

mit einer sozialen Funktion dieser Rufe. Tatsächlich konnte gezeigt werden, dass 50-kHz Rufe zu einem auf die Schallquelle hin ausgerichtetem Annäherungsverhalten führen (Studie IV). Diese Beobachtung steht jedoch im Widerspruch zu Untersuchungen, bei denen in Reaktion auf die Darbietung 50-kHz Rufen kein Annäherungsverhalten zu beobachten war (Burman et al., 2007; Endres et al., 2007). Mögliche Ursachen hierfür könnten Unterschiede in der verwendeten Technik oder die Platzierung des Lautsprechers sein. Die hier vorgelegten Befunde stehen aber in Einklang mit der Beobachtung, dass die Ausschaltung des Hörvermögens das Spielverhalten junger Ratten beeinflusst (Siviy & Panksepp, 1987), oder dass Ratten mehr Zeit mit Artgenossen verbringen, die viel vokalisieren, als mit Artgenossen, die wenig 50-kHz Rufe aussenden (Panksepp et al., 2002). Ferner konnte in einer Untersuchung, in welcher Ratten die Möglichkeit hatten, sich 50-kHz Vokalisationen mittels Durchbrechung einer Lichtschranke selbst zu verabreichen, ein positiver Anreizcharakter von 50-kHz Vokalisationen nachgewiesen werden (Burgdorf et al., im Druck). Ratten durchbrachen die Lichtschranke, welche mit der Präsentation von 50-kHz Rufen gekoppelt war, häufiger als eine Lichtschranke, die nicht mit der Präsentation von Vokalisationen gekoppelt war.

Auffällig ist hierbei die Übereinstimmung mit den an Mäusen gewonnenen Daten. So konnte beobachtet werden, dass die 70-kHz Rufe der Maus parallel zur sozialen Investigation auftreten (Maggio & Whitney, 1985). Beispielsweise korreliert die Zeit, welche die Tiere mit dem Beschnuppern der Partners verbringen, positiv mit den emittierten 70-kHz Rufe (Moles et al., 2007; Panksepp et al., 2007). Bemerkenswerterweise konnte ferner gezeigt werden, dass die Anzahl emittierter 70-kHz Rufen höher ist, wenn eine soziale Investigation wichtige Informationen liefern kann, wie etwa über ein unbekanntes Futter (Moles & D'Amato, 2000) oder den Status des Partners (Moles et al., 2007). In einer mit den vorgelegten Befunden in Einklang stehenden Interpretation wird spekuliert, dass die Emission von 70-kHz Rufen dazu dient, den Sozialkontakt ausreichend lange aufrecht zu erhalten, so dass genügend Zeit für eine ausführliche soziale Investigation gegeben ist (Moles & D'Amato, 2000). In Erweiterung der angenommenen Funktion der 70-kHz Rufe muss also davon ausgegangen werden, dass die von Pomerantz et al. (1983) im sexuellen Kontext durch 70-kHz Rufe beobachtete Induktion sozialer Annäherung auch für den nicht-sexuellen Kontext zutrifft.

Bemerkenswerterweise konnte beobachtet werden, dass juvenile Tiere stärker auf die Präsentation von 50-kHz Rufen reagieren als adulte Tiere (Studie IV). Dies stimmt mit der Tatsache überein, dass juvenile Tiere selbst eher 50-kHz Rufe aussenden (Panksepp &

Burgdorf, 1999). Beide Phänomene könnten auf eine mit dem Älterwerden einhergehende Abnahme des sozialen Interesses zurückgeführt werden (Salchner et al., 2004).

Die Annahme jedoch, dass die Frequenzmodulation ein zentrales Merkmal der 50-kHz Rufe darstellt (Burgdorf & Panksepp, 2006; Burgdorf et al., 2007; im Druck) konnte im Hinblick auf deren kommunikative Rolle nicht bestätigt werden. Sowohl juvenile als auch adulte Tiere zeigten Annäherungsverhalten auf artifiziell verfremdete 50-kHz Rufe ohne Frequenzmodulation (Studie IV). Die Hypothese, dass der affektive Zustand sich in der Ausprägung der Frequenzmodulation widerspiegelt, konnte insofern jedoch gestützt werden, als dass die nach Trennung vom Artgenossen auftretenden Rufe im Vergleich zu Rufen während des Kitzelns (Burgdorf et al., im Druck) häufig keine Frequenzmodulation aufwiesen (Studie III). Bemerkenswerterweise unterscheiden sich die Rufe jedoch nicht allein in der Ausprägung der Frequenzmodulation, sondern auch hinsichtlich der Frequenz (Burgdorf et al., im Druck). Eine mögliche Ursache für das Ausbleiben eines unterschiedlich stark ausgeprägten Annäherungsverhaltens in Abhängigkeit der Frequenzmodulation könnte also sein, dass nicht die Frequenzmodulation, sondern die Frequenz, bedeutende kommunikative Information enthält. Tatsächlich beobachteten Burgdorf et al. (im Druck) eine Präferenz für modulierte Rufe im Vergleich zu unmodulierten Rufen im Rahmen einer Selbstverabreichungsstudie unter Verwendung natürlicher Rufe. Zukünftige Untersuchungen werden zu prüfen haben, welche Rufmerkmale für den positiven Anreizcharakter dieser Vokalisationen verantwortlich sind. Unter Verwendung artifiziell erstellter Vokalisationsmodelle konnte beispielsweise bereits gezeigt werden, welche Rufmerkmale von 60-kHz Rufen der jungen Maus entscheidend für die Induktion des maternalen Eintrageverhaltens sind (Ehret, 1992; Ehret & Haack, 1981; 1982).

Der positive Anreizcharakter der 50-kHz Vokalisationen spiegelte sich jedoch nicht allein im gezeigten Annäherungsverhalten, sondern auch in der Aktivierung von Hirnregionen, wie etwa dem Nucleus accumbens (Studie V), die mit Belohnungsprozessen in Zusammenhang stehen (Schultz et al., 1997; Wise, 1996). Die im Nucleus accumbens beobachtete Aktivität steht in Übereinstimmung mit einer Studie zur Hirnaktivität beim Spielverhalten, wo ebenfalls eine erhöhte Aktivität im Nucleus accumbens beobachtet werden konnte (Gordon et al., 2002). Beim Spielverhalten treten im Allgemeinen 50-kHz Vokalisationen auf (Brunelli et al., 2006; Burgdorf et al., im Druck; Knutson et al., 1998). Die hier vorgelegten Befunde verweisen darauf, dass die dort beobachtete Aktivität in Folge des Spiels nur teilweise auf das Spiel selbst, zum anderen jedoch auf die Perzeption der 50-kHz Rufe zurückzuführen ist. In Ergänzung zur vorgelegten Arbeit über die in Reaktion auf die

Darbietung von 50-kHz Rufen induzierte Hirnaktivität, sollte vergleichend eine Untersuchung zur Hirnaktivität bei Produktion von 50-kHz Vokalisationen durchgeführt werden. Hierzu würde sich die experimentelle Nachahmung des Spiels junger Ratten durch Kitzeln anbieten, da hierfür nur ein Tier benötigt wird und somit gesichert ist, dass keine 50-kHz Rufe eines anderen Tieres die in der vorgelegten Studie V beschriebene Hirnaktivität auslöst. Ausgehend von Studien zur Hirnaktivität nach Verabreichung von Amphetamin (Robertson et al., 1991), welches bei der Ratte zu 50-kHz Vokalisationen führt (Burgdorf et al., 2001; im Druck; Knutson et al., 1999; Thompson et al., 2006), kann vermutet werden, dass auch während der Produktion der Nucleus accumbens aktiviert ist. Auf eine zentrale Bedeutung dopaminerger Neurotransmisson verweisen auch die Befunde, dass elektrische Stimulation dopaminerger Bahnen im Gehirn ebenfalls zu 50-kHz Vokalisationen führen (Burgdorf et al., 2000; 2007). Tatsächlich konnte gezeigt werden, dass nach elektrolytischer oder pharmakologischer Ausschaltung des ventralen Tegmentums 50-kHz Vokalisationen deutlich seltener auftraten, wie auch nach Gabe des D1/D2-Dopmain-Antagonisten Flupenthixol (Burgdorf et al., 2007). Es liegt daher auch nahe anzunehmen, dass die in Studie V beobachtete Aktivität im Nucleus accumbens auf dopaminerger Innervation beruht. Um dies experimentell zu prüfen, erscheint es sinnvoll die Tiere nach Verabreichung eines Dopamin-Antagonisten 50-kHz Rufen auszusetzen. Es wäre zu erwarten, dass die zuvor beobachtete Aktivität im Nucleus accumbens nicht länger zu vorzufinden ist.

Zusammenfassend kann gesagt werden, dass 50-kHz Vokalisationen einen der Aufrechterhaltung von Sozialkontakt dienliche Funktion haben. Die hier vorgelegten Untersuchungen als auch die Befunde an Mäusen (D'Amato & Moles, 2001; Maggio & Whitney, 1985; Moles & D'Amato, 2000; Sewell, 1970; Panksepp et al., 2007) zeigen, dass die soziale Funktion der Rufe nicht auf den sexuellen Kontext (Geyer et al., 1978; McIntosh et al., 1978; White & Barfield, 1987; 1989; 1990) beschränkt ist.

Angesichts der Stärke und der hohen Zuverlässigkeit mit welcher durch 50-kHz Rufe Annäherungsverhaltens induziert werden kann, erscheint es möglich, anhand dieser Methode die genetischen und neurochemischen Grundlagen sozialen Interesses zu untersuchen. So erscheint es vielversprechend zu prüfen, ob Blockierung der für das Sozialverhalten relevanten Neuropeptide Oxytozin und Vasopressin (zur Übersicht siehe: Bartz & Hollander, 2006; Lim & Young, 2006; Insel & Young, 2001) zur Reduktion des Annäherungsverhaltens führt. So konnte beispielsweise gezeigt werden, dass Knock-Out Mäuse ohne Oxytozin Defizite im Sozialverhalten, wie etwa eine soziale Amnesie, aufweisen (Ferguson et al., 2000). Außerdem emittieren Tiere, bei denen der Vasopressin1b-Rezeptor genetisch

ausgeschaltet wurde, während sozialen Interaktionen weniger 70-kHz Rufe im Erwachsenenalter (Scattoni et al., 2007). Bemerkenswerterweise unterscheiden sich diese Tiere auch hinsichtlich der isolations-induzierten Ultraschallvokalisation. Tiere ohne Oxytozin zeigen eine reduzierte Anzahl isolations-induzierter Ultraschallvokalisationen (Winslow et al., 2000) und Tiere ohne Vasopressin1b-Rezeptoren zeigen nicht den überlicherweise zu beobachtenden Anstieg an 60-kHz Rufen nach einem kurzen Kontakt mit der Mutter (Scattoni et al., 2007). Für die isolations-induzierte Ultraschallvokalisation ist ferner das Opioid-System von zentraler Bedeutung. So lässt sich die isolations-induzierte Ultraschallvokalisation etwa durch Opioid-Agonisten, wie etwa Morphium, absenken (Carden et al., 1996) und mu-opioid-Knock-Out Mäuse vokalisieren kaum in Isolation (Moles et al., 2004). Es ist bemerkenswert, dass auch das juvenile und adulte Sozialverhalten durch die Aktivität des Opioid-Systems beeinflussbar ist. Zahlreiche Untersuchungen zeigten die Involviertheit dieses Systems beispielsweise beim Spielverhalten (Vanderschuren et al., 1995a; 1995b; zur Übersicht: Vanderschuren et al., 1997). Außerdem ist bekannt, dass mu-opioid-Agonisten, wie Morphium oder DAMGO, die Auftretenshäufigkeit von 50-kHz Rufen erhöhen können, wohingegen der mu-opioid-Antagonist Naloxone das Rufverhalten hemmt (Burgdorf et al., 2001; 2007). Abgesehen von den genetischen und neurochemischen Grundlagen des Sozialverhaltens könnte die Erfassung des durch 50-kHz Rufe induzierten Annäherungsverhaltens helfen, den Einfluss von Umweltfaktoren, wie beispielsweise den maternaler Fürsorge, auf das adulte soziale Interesse zu bestimmen.

Im Vergleich mit anderen Verfahren zur Bestimmung des sozialen Interesses zeichnet sich die Erfassung des durch 50-kHz Vokalisationen induzierten Annäherungsverhaltens dadurch aus, dass ein exakt zu bestimmender Stimulus eingesetzt wird, welcher zudem experimentell hinsichtlich seiner Anreizstärke verändert werden kann, wohingegen bei den anderen Verfahren meist zwei Tiere, wie etwa beim Spiel, verwendet werden, wobei eines als Stimulus dient, weshalb es augenscheinlich bei diesen interaktionistisch aufgebauten Verfahren unmöglich ist, den Stimulus konstant zu halten oder auch nur exakt zu bestimmen. Die Untersuchung des durch 50-kHz Vokalisationen ausgelösten sozialen Annäherungsverhaltens erscheint daher besser geeignet zu sein, um zu einem tieferen Verständnis der genetischen und neurochemischen Grundlagen von psychischen Störungen zu gelangen, welche durch Auffälligkeiten im Sozialverhalten gekennzeichnet sind, wie etwa Depression und Autismus.

ZUSAMMENFASSUNG

Mäuse und Ratten verfügen über die Fähigkeit, Ultraschallvokalisationen auszusenden. Diese Ultraschallvokalisationen treten in motivational relevanten Kontexten auf. Sie sind für die biopsychologische Forschung von großer Bedeutung, da die Tiere in Abhängigkeit ihres motivational-affektiven Zustandes unterschiedliche Vokalisationen emittieren und somit Einblicke in die Grundlagen von Emotion und Motivation gewähren können. Über die funktionale Bedeutung dieser Ultraschallvokalisationen besteht jedoch Unklarheit. In den vorgelegten Arbeiten sollte daher geprüft werden, inwiefern es sich bei den Ultraschallvokalisationen um kommunikative Signale des motivational-affektiven Zustands handelt. Hierzu wurde zum einen geprüft, welche Bedeutung soziale Faktoren, wie maternale Fürsorge oder An- beziehungsweise Abwesenheit eines Artgenossen, für den Sender, das heißt für die Produktion von Rufen, haben. Zum anderen wurde geprüft, welchen Einfluss die Produktion von Rufen auf den Empfänger hat. Es konnte gezeigt werden, dass sich die erfahrene maternale Pflege auf die Produktion isolations-induzierter Vokalisationen im Jungtier als auch auf die im Erwachsenenalter auftretenden 22-kHz Vokalisationen auswirkt, wohingegen die aktuelle An- beziehungsweise Abwesenheit eines Artgenossen die Emission von 22-kHz Rufen nicht beeinflusst. Die Effekte maternaler Fürsorge sind möglicherweise über Beeinflussung des Aufteretensverhältnisses von aktiven und passiven Bewältigungsstrategien in aversiven Situationen vermittelt. Ferner konnte gezeigt werden, dass obwohl die An- beziehungsweise Abwesenheit eines Artgenossen keinen steigernden Einfluss auf die Produktion von 22-kHz Rufen hat, diese dennoch angst-ähnliches Verhalten beim Empfänger induzieren können. Im Gegensatz zu der durch 22-kHz Rufe induzierten lokomotorischen Inhibition, steigern 50-kHz Rufe die lokomotorische Aktivität und führen zu Annäherungsverhalten. In Übereinstimmung mit den entgegengesetzten Verhaltensreaktion aktivieren 22-kHz Rufe Hirnstrukturen, die an der Regulation von Angst und Furcht beteiligt sind, wohingegen 50-kHz Rufe Strukturen aktivieren, die mit Belohnungsprozessen in Zusammenhang stehen. Die vorgelegten Arbeiten stützen demnach die Hypothese, dass Ultraschallvokalisationen als kommunikative Signale des motivational-affektiven Zustands dienen. Die hier etablierten Verhaltensparadigmen werden es zukünftig ermöglichen die biopsychologischen Grundlagen verschiedener Aspekte von Sozialverhalten zu untersuchen. So kann beispielsweise die durch die Präsentation von 50-kHz Vokalisationen induzierte Verhaltensreaktion der Tiere genutzt werden, um die genetischen und neurochemischen Grundlagen sozialen Annäherungsverhaltens zu beschreiben und so möglicherweise Einblick in die Pathomechanismen von psychischen Störungen gewähren, die durch Defizite im Sozialverhalten gekennzeichnet sind.

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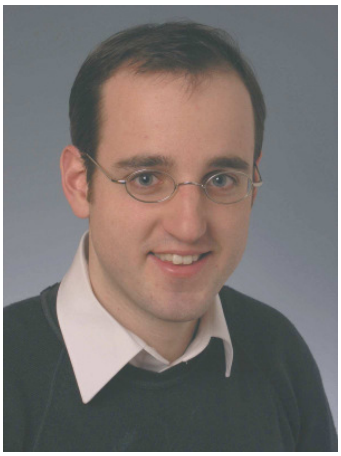

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ERKLÄRUNG

Ich versichere, dass ich meine Dissertation

ULTRASCHALLVOKALISATIONEN BEI MAUS UND RATTE

–

KOMMUNIKATIVE SIGNALE DES MOTIVATIONAL-AFFEKTIVEN ZUSTANDS?

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

_____ _____

Ort/Datum Unterschrift